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A COMPARATIVE MEASUREMENT OF IN VITRO DIGESTIBILITY AND VOLATILE FATTY ACIDS FROM NUI/WHITE CLOVER AND MATUA/WHITE CLOVER GRASS SWARDS

A thesis submitted in partial fulfilment
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A COMPARATIVE MEASUREMENT OF IN VITRO DIGESTIBILITY AND VOLATILE FATTY ACIDS FROM NUI/WHITE CLOVER AND MATUA/WHITE CLOVER GRASS SWARDS

by

W. Joe

Pasture yield and In Vitro Digestibilities (IVD) were measured for Matua/white clover and Nui/white clover swards growing in Canterbury. Samples were collected at two weekly intervals over 10 different periods of regrowth from defoliation in May, until September. Each cultivar had two starting dates one week apart. The volatile fatty acids (VFA's) from the fermentations were separated for composition, and the total VFA produced was examined for linkage to IVD.

The dry matter yield accumulation of herbage produced a difference of 247 kg/ha dry matter between cultivars. The cultivar means were 1247 kg/ha for Matua and 1494 kg/ha for Nui. Regrowth after defoliation to soil level showed Nui had recovered to 31.3% grass of the sward composition by week 2, but Matua in comparison had only reached 3.8%. Clover proportion from the Nui sward was higher than from the Matua sward throughout the collection period. The maximum difference was 29.2%, and this occurred 6 weeks after defoliation, when Matua clover% was 2.2% and Nui clover% was 31.4%.

The mean IVD values arising from different cultivars were Matua 39.5% and Nui 60.9%. The period of growth accumulation caused the mean IVD to drop from an initial 40.5% at week 2, to a minimum of 26.4% at week 6, and then rose continuously to 71.9% at week 20. The starting date also had a significant effect on the IVD. The mean IVD's were 51.8% for start date A, and 48.5% for start date B.

The GLC separation of VFA's after in vitro fermentation of samples for 48 hours could detect differences of individual VFA composition, and the total VFA produced. The mean total VFA concentration over the 20 weeks were 212 mg/100ml for Matua, and 321 mg/100ml for Nui. Start date A produced 258 mg/100ml and start date B produced 275 mg/100ml. There was a minimum at 8 weeks from defoliation which rose to 378 mg/100ml at week 20. Interaction occurred between all three factors.

The composition of Matua acetic acid was 63.8 mmol% at week 2 and had dropped to 57.7 mmol% by week 18. Nui was 63.4 mmol% and 59.5% respectively. Matua propionic acid was 24.1 mmol% at week 2 and rose to 29.3% at week 18. Nui was 24.8% and 28.0 mmol% respectively. The acetic/propionic acid ratio rose to a maximum at week 6, and this was coincident with the lowest yield and lowest IVD. The start dates had different effects on the Matua and Nui cultivars.

The total VFA's produced the following correlation coefficients to IVD : $r^2 = 0.98$ for Matua and $r^2 = 0.87$ for Nui. The change of VFA composition due to IVD was also evident.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Herbivores have evolved to utilise carbohydrates although the animals lack the enzymes to attack the beta 1-4 glucosidic linkage of cellulose. Breakdown of structural polysaccharides is acheived through a symbiotic relationship with rumen micro-organisms which secrete the necessary enzymes.

The host animal provides an anaerobic environment of stable temperature for reproduction of the microorganisms. In return these microorganisms benefit the host through hydrolysis of plant polymers to products which the animal can utilise. Synthesis of vitamins frees the animal from diet vitamin dependence (except vitamins A and E), and synthesis of protein also occurs.

Two basic kinds of herbivores exist, hindgut fermenters in which the process occurs in the large intestine eg. horses and rabbits, and foregut fermenters eg. camels and sheep. Essentially the difference between monogastrics and ruminants lie in their overall ability to digest structural carbohydrates commonly referred to as 'fibre'.

Fibre also contains non-carbohydrate constituents such as lignin, pectin, gum and mucilage. Plant carbohydrate starches, sugars and fructosans occur in the soluble portion. Cellulose and hemicellulose make up the structural fraction. Nutritional quality of a diet can be measured as the proportion digested.

Various laboratory methods have been developed to determine the fibre partitions eg. Weende crude fibre, normal acid fibre (NAF), acid detergent fibre (ADF), neutral detergent fibre (NDF). Each procedure solubilizes and removes different component combinations leading to different results for the analyses. Hence each method is a modification to circumvent inadequacies of a former method.

Since these are all poor predictors of ruminant digestibility, a method based on digestion of structural carbohydrate by cellulase, cellobiase, xylanase and pectinolytic substances was developed by Tilley and Terry (1963).

The first 48 hours of digestion occurs through incubation with rumen liqour in vitro, simulating digestion by the rumen of the animal. Subsequently HCl and pepsin are added to the incubation mixture to simulate the processes which occur in the abomasum of the ruminant.

The bacteria carry out digestion by moving through the rumen and attaching themselves to the substrate. Then the bacteria secrete extra-cellular carbohydrate digesting enzymes.

Fibre digesting bacteria have different cellulose digestion capabilities resulting from different proportions of enzymes produced. Fibre digestion is a complex interaction of amounts of enzymes produced, types of enzymes, activities of each enzyme, and substrate composition.

Differences in herbage fermentation may arise from two sources: (1) plant variety, and/or (2) composition of the samples. The main differences between grasses and clovers are: (1) pectin is present in significant amounts in clovers but not in grasses; (2) soluble reserve polysaccharides in grasses are fructosans while clovers contain starch; and (3) and clovers tend to contain less cell wall carbohydrates (Bailey, 1964).

Cellulose and hemicellulose favour a fermentation with acetic acid predominant amongst the end products while soluble carbohydrates such as fructosan and starch lead to a higher proportion of propionic and butyric acids. Rates of fermentation are dependent on accessibility of microbial enzymes and therefore soluble carbohydrates are more readily fermentable.

Thus clover in a grass pasture should promote a faster rate of passage. Johns et al., (1963) found animals from NZ 'grasslands Manawa' (Lolium hybridium) Hausskn, plus white clover (Trifolium repens) pastures had higher proportions of propionic and butyric acids compared to animals from pure perennial ryegrass pastures.

Because the volatile fatty acids (VFA's) may enter separate metabolic pathways, the amount and composition of VFA's will affect animal performance. Of the animal total energy intake, 70-80 percent of this is provided through VFA's, acetic and propionic being the two major sources.

Acetic is utilised for lipogenesis and metabolised for energy through the tricarboxylic acid cycle. Propionic is the most important precursor for glucose, being synthesized in the liver and provides approximately 80 percent of the glucose for lactose in milk. In lactation, increase of acetic level elevates milk yield and fat content while increase of propionic reduces milk fat content.

Prairie grass 'Grasslands Matua' Bromus Catharticus is a cultivar bred by Grasslands Division, DSIR, Palmerston North and introduced in January 1973 (Rumball, 1974). Although productivity comparisons under close mowing have been made by Rys et al. (1977) and by Baars and Cranston (1977), no comprehensive information on *in vitro* digestibility has been published.

NZ 'grasslands Nui' is a polycrossed perennial ryegrass (Lolium perenne L.) bred from elite genotypes selected for productivity and rust resistance. It was placed on the New Zealand list of acceptable cultivars on January 1973. Trials at 3 locations has shown it to consistently produce greater yield than other ryegrasses, especially after dry periods (Armstrong, 1977).

The work reported here provides a comparative measurement of the quality of growth accumulation of Matua and Nui for periods from 2 weeks to 20 weeks. This was started in Autumn, and finished in Spring following different periods of regrowth. The in vitro digestibilities (IVD) were determined and the VFA's from the first stage of digestion were separated to determine the ratio of the 2 major VFA's, acetic and propionic acids. The total VFA concentrations from the in vitro fermentations were examined for linkage to IVD.

A parallel objective was to develop a system for analysis of VFA's at Lincoln College. A method for the preparation of VFA samples was devised, and a reliable method for the quantitative determination of VFA's by gas-liquid chromatography was set-up.

CHAPTER 2

REVIEW OF LITERATURE

In this review of literature,

1. aspects of fermentation are reviewed with their relevance to animal metabolism and utilisation.

2. various aspects of procedures employed for similar analyses are discussed. Sources of problems and limitations are identified.

The review of results reported by authors have different parameter values. To make comparisons clearer, the results have a tabulated format for easy cross-reference.

2.1 RUMEN FERMENTATION

A symbiotic relationship exists between the ruminant and the bacteria which are mainly acid producing anaerobes. Cellulase produced by the bacteria degrade cellulose and hemicellulose in the rumen , which precedes the true stomach the abomasum.

There is considerable variation amongst bacteria regarding the substrates fermented. The most important cellulose utilizers, Ruminococcus and Bacteroides, attack only cellulose, cellobiose and one or two other substrates. Most strains of Ruminococcus do not attack free xylose or glucose, but ferment xylan whereas Butyrivibrio attack a range of carbohydrates including mono-, di-, and polysaccharides.

In general, micro-organisms are only loosely associated with food particles, though spatial relationships of different organisms may not be random. Some may hold more favourable positions in relation to food particles (Hungate, 1966). Readily soluble carbohydrates and sugars are rapidly fermented in the rumen, but the breakdown of cellulose is much slower.

Amongst the large number of factors which affect fermentation of carbohydrates, the most important are probably the availability of protein and non-protein nitrogen, and the extent of lignification. Lignins may physically prevent the enzymes of the organisms from attacking the cellulose and hemicellulose, while the availability of nitrogen affects the ability of the organisms to grow and reproduce.

The conversion of substrate to VFA occurs in 3 stages:

1. starch and cellulose are fragmented into maltose and cellobiose, which are eventually broken down into glucose. Pectin and hemicellulose are broken down through their oligosaccharides into several other sugars.

2. glucose and fructose then enter the Emden-Meyerhof pathway to produce the key intermediate pyruvate.

3. specific enzymes in the bacteria then dictate the conversion of pyruvate to the end products.

Bacterial interaction may occur as most bacterial species are capable of utilizing several substrates. These may be the end products or the intermediates of the other bacteria and hence a change of substrate may have indirect or secondary effects on bacterial population proportion. Therefore the quantities of VFA's produced and the bacterial cells synthesized in the rumen are related.

The diet can therefore influence the rumen environment which in turn alters the substrate availability to the micro-organisms. As a consequence the pattern of microbial species will be affected, which in turn will affect the balance of enzymes, pathways and hence composition of the VFA's.

Attachment of the bacteria to the substrate invovles London-Van der Waals forces and electrostatic forces. The extra-cellular cellulases operate in a synergistic fashion. Reese et al. (1950), proposed that a Cl cellulase carried out a preliminary hydrolysis by activating or disaggregating the cellulose chains in preparation for hydrolysis by the Cx enzymes. A mechanism for this hydrolysis was proposed by Pettersen (1975). The rumen microbial degradation of plant cell walls examined by electron microscopy has been reported by Akin (1982).

Acetic and propionic are the two major fatty acids produced during fermentation. They are used for different metabolic processes: adipose tissue, milk fat synthesis, energy production, glucose synthesis, production of ketone bodies. The relative proportions of each VFA alter the energy status of the animal and therefore manipulation of the VFA proportions can alter the utilization efficiency.

Fibrous feeds of low digestibility usually give ratios of greater than 7:2 for acetic:propionic; the ratio tending to favour propionic acid with soluble carbohydrates, reaching unity on a mainly concentrate diet. As the rumen pH falls, the acetic acid level falls and the propionic acid level rises.

This has been confirmed by buffering the in vitro system to 3 differnt pH levels of 6.0, 6.5, 7.0 (Raymond and Terry, 1966)

2.1.1 Substrate Influence On IVD

The change of IVD with sward growth, occurs because of two fundamental reasons:

1. different digestibility between chemical constituents (cellulose, hemicellulose, lignin)
2. composition change of chemical constituents in the plants.

2.1.1.1 Plant Composition Changes -

The rates of fermentation depend on the accessibilty of rumen microbial enzymes to the substrates. Soluble carbohydrates once released from the plant cells are much more rapidly fermented than the structural cell wall carbohydrates.

The fermentation rates from various carbohydrate fractions from fresh red clover are given in the table 2.1.2.1 below.

TABLE 2.1.2.1 : Carbohydrate fermentation rates.

carbohydrate	%loss 3 hr after end of feed
soluble	100
fructosan	-
starch	71
pectin	35
hemicellulose	22
cellulose	15

The carbohydrate composition of soluble sugars + water soluble polysaccharides + pectin / hemicellulose + cellulose is 1.58 for clover and 0.78 for ryegrasss. This leads to higher digestibility, rate of passage, intake, and fermentation rate for bulked samples containing a greater content of clover.

Several authors have investigated the relationship of plant parts to digestibility. The effect of treatment on botanical composition and composition of plant parts were measured.

Harkness and Alexander (1969) found highest yields of digestible organic matter with less frequent cutting regimes, but mean digestibility of the herbage was lower.

Most of the IVD variation was due to the acid-pepsin digestible fraction, the three major constituents being crude protein, hot water soluble carbohydrates, and hot water soluble dry matter. (Tilley and Terry, 1969).

Walters et al. (1967) found that the stage of growth, leafiness and dead matter accumulation partly accounted for IVD differences between species, but almost entirely accounted for difference between varieties within species. Seasonal patterns arose from ageing due to:

1. gradual decline of leaf IVD
2. increasingly rapid decline of stem IVD
3. increasing proportion of stem
4. increasing proportion of dead material

Wilman et al. (1977) reported similar results. IVD decline was due to decline in cell ~~wall~~ content, and the decline in cell wall digestibility. The cell contents were almost entirely digestible, while the cell wall digestibility was related to the extent of cell wall lignification. **In white clover, the decline in cell contents was entirely responsible for the decline in digestibility.**

In lucerne the change was intermediate between white clover and ryegrasss. The leaves of lucerne had similar contents of cellulose and hemicellulose at all stages of maturity. In contrast, stems become progressively more

fibrous and their digestiblity decreased. This lead to the overall digestibility of the whole plant falling with maturity.

Fletcher (1976) found that lucerne had a relatively constant leaf IVD irrespective of maturity or season, but stem IVD decreased to below 50% for long regrowth over summer. Leaf /stem ratio was a good indication of the overall IVD, and for the whole plant this varied from 57% to 81.4% . Rapid decline in IVD occurred at flowering onset, although this rose again after flowering ceased.

2.1.1.2 Chemical Constituent IVD Changes -

Legumes accumulate mainly sucrose, tropical grasses store starches, while fructosans are mainly stored in temperate grasses. Perennial ryegrasses contain approximately 12% soluble carbohydrates in the leaf and 25% in the stem with an average of 13% in the total herbage.

Investigation into the chemical constituents has established that hexosans (cellulose), pentosans (hemicellulose), change very little in the leaf with maturity, but nearly doubles in the stem. The two materials together, make up 50% - 75% of the total plant fibre. Hexosan decreased as the plant matured, while total content of digestible hexosan was nearly constant over the whole range of maturities studied.

Young grass stems have a very high level of soluble carbohydrate, higher than either leaf blade or leaf sheath, leading to a much greater reduction in stem digestibility as soluble carbohydrate is lost with increasing maturity.

Wilman and Daly (1974) in a study of regrowth of Italian ryegrass from 2 to 14 weeks, found the reduction of IVD was due to the increase in the proportion of the cell wall and the reduction in the digestibility of the cell wall.

Cell wall digestibility reduction was due to reduced cellulose and hemicellulose IVD, and both were highly correlated to the proportion of lignin in the cell wall. The proportion of digestible cell wall is not constant, being highest after 4-6 weeks. The ratio of cellulose to hemicellulose averaged 1:0.89, and hemicellulose was more digestible than cellulose. The rate of increase of cell contents appeared to diminish from about the third week onwards.

Other authors have found cellulose to be more digestible than hemicellulose. (Jarrige and Minson, 1964; Waite et al., 1964).

In contrast Tilley, Terry, Deriaz and Oulten (1969) reported that the amount of digestible cellulose, hexosan and pentosan were not greatly affected by the species, nor by maturity, but marked differences occurred between grasses and legumes. The rate of digestion was independent of the total amount of structural material present. As perennial ryegrass matured the following changes occurred:

1. hexosan content rose from 23% to 29%
2. IVD drop from 85% to 54%
3. content of digestible hexosan varied from 16% to 20% with no clear trend.

The principal storage polysaccharide in temperate grasses are fructans, of which there are 2 classes:

1. levans with beta 2-6 linkage
2. inulin with beta 2-1 linkage

The other classes of constituents affecting digestibility are: cellulose, hemicelluloses, and lignin (although not a

carbohydrate, it has a dominant effect on IVD). Cellulose which makes up a major portion of the cell wall, is a linear homopolymer with beta 1-4 linked glucopranosyl residues with a chain length of 1000 units. Hydrogen bonding occurs between the chains forming a regular tightly packed parallel structure.

Hemicelluloses are heteropolymers, the xylans and glucomannans being the most abundant. They have chain lengths similar to cellulose and also beta 1-4 linkages.

Lignin is an inert polymer based on phenylpropane residues which act as a cementing agent in the cell wall. Morrison (1974) found evidence of covalent binding of lignin to hemicellulose in grasses.

The digestibility of cell wall carbohydrates decline with maturity, with hemicellulose declining much more rapidly than cellulose. Chemically the structures do not alter with age, and therefore should be of similar digestibility in both young and mature plants. However plant parts decline in digestibility, and two theories have been advanced for the decreasing digestibility:

1. physical shielding from the enzyme attack by the lignin, (fine grinding increases IVD by disrupting the lignin)
2. young grasses are sparse in lignin, and therefore more easily recognised by the hydrolases.

As grasses mature, the content of lignin, cellulose and hemicellulose increases as a proportion of the total organic matter. But as a proportion of the cell wall, cellulose decreases and hemicellulose increases.

Lignin-hemicellulose complexes need a cell wall modifying enzyme prior to polysaccharide degradation. As the lignin rises, the extent of hydrolysis declines. Hemicelluloses act extracellularly, being found in cell free rumen fluid.

2.1.2 VFA Metabolism

The VFA's are absorbed from the rumen by transport through the rumen epithelium, at a rate inversely related to their chain length. It has been shown that pH also has a profound effect on the absorption rates.

pH 7.5 acetate > propionate > butyrate

pH 5.8 Butyric > propionic > acetic

The VFA absorption increases with decrease of pH of the rumen contents, indicating a greater permeability of the tissue to the undissociated form of the acids.

2.1.2.1 Acetic Acid -

A small part of the acetic acid is converted to ketone bodies in the rumen epithelium, but the major part is passed onto the liver unchanged where a small part is further converted to ketone bodies. The rest enters the peripheral circulation. Free acetic acid can undergo activation in the cell cytoplasm resulting in acetyl-CoA. From this point it follows the pathways open to acetyl-CoA and hence provide a source of energy leading to fatty acid sythesis or to the production of ketone bodies. It cannot directly produce intermediates of the tricarboxylic acid cycle (TCA).

More than 80% of ketone bodies occurring in sheep are beta-hydroxybutyrate, the remainder being acetone and acetoacetate. Acetoacetate and beta-hydroxybutyrate compete with glucose as substrates for oxidative metabolism via the TCA cycle. The kidney, heart and spleen are the most active in sheep. Acetoacetate stimulates glucogenesis through activation of pyruvate carboxylase. Ketogenesis is affected by oxaloacetate as it is the intermediate between pyruvate and phosphopyruvate. If there is a shortage of oxaloacetate to allow utilization of acetyl-CoA via the TCA cycle, beta oxidation of long chain fatty acids occurs leading to accumulation of acetyl-CoA which leads to formation of ketone bodies, acetoacetate, beta-hydroxybutyrate and

acetoacetyl-CoA.

2.1.2.2 Propionic Acid -

Some of propionic acid is also converted to ketone bodies in the rumen epithelium, the rest passes to the liver where it is either oxidised or converted to glucose. The path of propionate into the TCA cycle involves CO_2 fixation of propionyl-CoA to methylmalonyl-CoA carboxylase, and is subsequently converted to succinate.

Propionate is an odd chain fatty acid which is rare in animal metabolism. It is taken by the liver from the portal blood and is glucogenic, producing about half the glucose requirements of the ruminant. The rest is contributed by the amino acids. This sharing of energy contribution cannot be performed by even chain fatty acids. Valeric acid is another odd chain fatty acid and also follows this path after loss of 2 carbon units as acetyl-CoA.

2.1.3 VFA Utilization

Calorimetric measurements by Blaxter (1962) showed the effiency of fat synthesis was only 25-30% on all roughage diets. The rumen contained 74% acetic acid on a molar basis, while concentrates produced rumen liqour with 45 molar% acetic acid. This increased fat synthesis to greater than 60%. Consequently higher proportions of propionic acid leads to more efficient lipogenesis since acetic and propionic acids are inversely related. Different mixtures of VFA's are utilized at about the same efficiency at maintenance. Calorimetric measurements of metabolizable energy for fat synthesis above maintenance showed it decreased as crude fibre increased.

Investigations by acetate labelling has demonstrated that acetic acid could be oxidised by the mammalian heart (Barcroft, 1946), is a source of carbons for fatty acids in milk from C4 to C16, and also for fatty acids in adipose

tissues. VFA's, despite composition being changed by infusion into the rumen, always had lower utilization efficiency than glucose as a source of maintenance energy. Blaxter (1962) showed that acetic acid by itself had a maintenance utilization efficiency of 59.2%. However this rose to 87.2% for a mixture of acetic/propionic/butyric in molar proportions of 25/45/30 respectively.

2.1.3.1 VFA's In Lactation -

Linzell (1960) demonstrated that decreasing acetic acid reduced milk fat content, while Tyznik and Allen (1951) showed the converse was true that when acetic acid was increased, milk fat could be increased.

Increasing propionic acid level led to a decrease in energy secreted as milk and also promoted adipose tissue synthesis (Thomas and Clapperton, 1972), while Reid (1950) observed a depressed secretion of the longer chain fatty acid C16 and C18.

Powell (1941) indirectly demonstrated this by a very low fibre content diet (higher propionic acid) which caused a depression of milk fat. Elliot and Loosli (1959) found the most efficient secretion of milk occurred at 22% propionic and 59% acetic acid molar proportions. Depressions of milk fat can be detected when molar proportions of propionic acid in the rumen liqour rise to 25% and are severe at 30%.

In summary,

1. increase of acetic acid increases milk yield and milk fat content. The converse is also true.

2. increase of propionic acid increases adipose tissue synthesis.

3. acetic and propionic acids are inversely related.

The quantitative determination of VFA's produced by in vitro fermentation may therefore provide important information on the influence of metabolism and utilisation on animal performance. This complements the less direct information of herbage quality provided by IVD.

2.2 ANALYSIS OF SAMPLES

2.2.1 Preparation Of Samples

Differences of results from sample preparation can arise from two main sources. (1) drying of herbage, and (2) grinding of sample.

Temperature and time are the parameters which can be altered for drying. Vacuum freeze drying relies on reducing pressure to below the triple point of water. At low pressure, temperature can be reduced while removing water from the sample. Forced air oven drying relies on increasing the temperature to remove moisture from the sample by evaporation. While the high temperature of oven drying denatures respiration enzymes, it also causes loss of soluble carbohydrates from heat. Freeze drying however, does not cause soluble carbohydrate loss, but more residual moisture is retained and the enzymes are not deactivated.

Waite and Boyd (1953) found negligible differences in hexose, sucrose and fructosan percentages when perennial ryegrass herbage was dried at 105-110 °C for 45 minutes in a forced draught oven when compared to fresh tissue. Deriaz (1961) also found little difference in water soluble carbohydrate percentages of three different temperate origin grasses dried at 100 °C in a forced draught oven as compared to fresh tissue on a dry weight basis.

However, Laidlaw and Wylam (1952) found a reduction in fructose, glucose and sucrose percentages and an increase in fructosan percentages in ryegrass dried at 105 °C compared with fresh freeze dried tissue.

This may be explained by the investigations of Link (1925), and Link and Tottingham (1923) which showed few heat drying processes were suitable for all kinds of tissues; drying at 60-80 °C were suitable for some tissues, but carbohydrate losses occurred from as low as 60 °C.

Rapid heat drying to reduce the time that enzyme activity can occur has been stated by many investigators. Jarrige (1954), Raguse and Smith (1965), Salo and Kotilainen (1970), and Smith (1969).

A recent review of the influence of drying and storage conditions on nonstructural carbohydrate analysis, has been carried out by Smith (1973).

Storage of samples should not present any problems if they are kept in airtight containers with a moisture content of less than 4% and in addition refrigeration will reduce thermal reactions. (Collins and Shorland, 1945)

The most acceptable heat drying regime is a short period of one hour or less at 100 °C and thereafter at 60-70 °C until sample weight is constant. (Smith, 1973) (Temperatures above 70 °C will stop bacterial action. Schmid, 1970).

Therefore samples containing a larger proportion of soluble carbohydrate are less suitable for heat drying due to its contribution to dry matter losses. Time should be kept as short as possible and tissue should be spread out in porous containers to expose largest surface area to enhance drying rate. Criteria for drying are a compromise of: (1) a temperature high enough to destroy enzyme activity, (2) and a temperature low enough to reduce the losses of soluble carbohydrates and the formation of artifact lignin through

protein-sugar reactions (via the non enzymic browning reactions Van Soest, 1965).

The effect of drying on IVD has been researched by several authours. A comparison of the effect of different drying methods on IVD values has been carried out by Penning, Barnes and Valderrabano (1977). Their results are shown in table 2.2.1A

Table 2.2.1A: comparison of drying methods
Penning, Barnes and Valderrabano (1977)

HERBAGE	DRYING METHOD OD	MWD	MWD50	FD	MEAN
Ryegrass 1	71.1	72.9	74.1	76.6	73.3
Clover	68.2	70.4	68.5	72.4	69.9
Ryegrass 2	74.3	77.0	78.7	81.7	77.9
Mean	71.2	73.4	73.8	76.9	73.7

OD = 100 °C for 16 hours
MWD = microwave drying to constant weight
MWD50 = microwave for 2 minutes then oven dried for 16 hours at 50 °C
FD = feeze drying

A comparison of the drying methods is summarised in table 2.2.1B

Table 2.2.1B : Comparison of drying methods

AUTHOR : Tilley and Terry (1963)
method 1: OD 40 or 100 °C
method 2: FD
comment: IVD same except when dried for longer than 4 days

AUTHOR : Grant & Campbell (1978)
method 1: OD 45 °C
method 2: FD
comment: OD had lower IVD due to Maillard non-enzymic browning reaction above 50 °C. Not all species were equally affected.

AUTHOR : Johnson et al.
method 1: OD
method 2: Fresh herbage
comment: OD had lower IVD

AUTHOR : Noller et al. (1966)
method 1: OD 60 or 80 °C
method 2: Lyophilized
comment: Lyophilized samples had higher organic acids, dry matter and IVD.

AUTHOR : Schmid et al.
method 1: OD 16 hours
method 2: OD 72 hours
comment: 72 hours had considerably lower IVD, therefore chemical analyses should not be carried out, but variety rankings are not changed.

AUTHOR : Burns et al.
method 1: OD
method 2: FD
comment: FD higher in water soluble N, lower total N, more soluble carbohydrates.

AUTHOR : Cochrane & Brown (1974)
method 1: OD 60, 80, 100 °C for 24 hours.
method 2: Ground frozen herbage
comment: reduced soluble CHO by 29.7% , reduced IVD 5.3% , 24 hours storage prior to drying did not affect dry matter, crude protein or IVD. Soluble CHO concentration was lower.

OD = Oven drying
FD = Freeze drying

Sharkey (1970) also recorded lower crude protein percentages with higher oven drying temperatures. Thomas and Armstrong (1949) and Van Soest (1965) found increased apparent lignin content at higher drying temperatures. Burns (1966) showed that this was accompanied by reduced water or detergent solubilty of nitrogenous compounds. This was suggested as the reason for lower IVD by Cochrane and Brown (1974).

Jones and Bailey (1972) confirmed that oven drying resulted in lower levels of soluble carbohydrate and higher levels of detergent insoluble nitrogen and hence cell wall constituents and lignin. However the percentage hemicellulose hydrolysed by rumen hemicellulase indicate essentially no difference between oven dried and freeze dried samples. Furthermore, hydrolysis of total polysaccharide in cell walls by hemicellulase plus cellulase mixture showed a negligible difference between oven and freeze drying. Insoluble nitrogen, presumably heat denatured protein, was at least 80% digested. It did not hinder access of carbohydrases to the polysaccharides.

Thus higher IVD values may arise from two sources:

1. freeze dried herbage will contain more residual or colloidal bound water.

2. soluble carbohydrates are almost entirely digested. Loss on drying will lead to a greater proportion of indigestible material in the dry matter, consequently leading to lower IVD values.

Tilley and Terry (1963) found that fine grinding greatly increased IVD values, being most marked in samples of initially low digestiblity. Physical form of a diet can influence the rate and duration of fermentation, and the number and types of microbes. The mean retention time was longer at a lower level of feeding (Alwash and Thomas, 1974). Fine grinding is thought to

disrupt cells and partially delignify fibres in forages increasing the available substrate surface area for fermentation.

2.2.2 Methods Of Measurement

Nutritional quality of feed will affect animal intake, digestiblity and supply of utilizable substances to the animal. Palatibilty will influence amount and type of feed ingested. This in turn will affect the retention time of the digesta in the rumen, and consequently the extent to which the substrate is digested.

The measurement of digestibility can be grouped into four categories. (1) chemical (2) animal (3)enzyme cellulase (4) microbiological in vitro.

2.2.2.1 Chemical Methods -

These are used to determine chemical composition as an estimate of forage digestibility. The Weende crude fibre analysis was developed by Henneberg and Stohmann (1860). This divides digestible organic matter into crude protein, crude fibre, crude fat and nitrogen free extract. Subsequent modification has been made to this system to improve it as a predictor of digestibility. To measure cell wall and cell components, Van Soest (1963) developed the acid detergent fibre procedure (ADF), and Van Soest and Wine (1967) developed the neutral detergent fibre (NDF).

Later, lignin and cellulose were used as estimators since the former is almost completely indigestible but ruminant bacteria partially digest cellulose and hemicellulose, and therefore they are not reliable estimators.

Aerts et al. (1977) made a comparison of laboratory methods for predicting organic matter digestibility (DOM) of forages (table 2.2.2.1). Weende crude fibre, ADF and NDF differed in their ability as predictors depending on the sample.

Table 2.2.2.1 : Correlation of chemical methods to DOM

METHOD	GRASS HAY r^2	SILAGE r^2	PELLETS r^2
WCF	0.35	0.68	0.62
ADF	0.41	0.66	0.32
NDF	0.53	0.65	0.68

Hi Koh Oh (1966) concluded that lignin gave the most satisfactory correlation within forage species, but none of the chemical components nor solubilty methods should be used for comparisons of different species.

Carlier (1976) reported digestibility of protein to be 94.8% in fresh grass and 80.9% in normal hay. It was concluded that a change in cell wall content (more crude protein and less soluble carbohydrate in response to nitrogen fertilizer dressing) would not have effect on protein digestibility. The protein digestibility would not change with the stage of growth of the grass, and does not change very much from one forage to another.

Johnson, Moore and Zank (1961) have published a method for determination of lignin in small wood samples. Lignin is a complex aromatic polymer which occurs in plant cell walls in close association with cellulose and hemicellulose polysaccharides. Progressive lignification is considered to be a major cause in the decline of digestibility as the herbage matures, and it is the grass component of the bulked herbage sample which is the most readily measured by chemical means. It also gave the best correlation to IVD.

Morrison (1972) adapted this method to measure lignin in herbage samples as a predictor of IVD. This method involves the prior removal of phenolic materials in dried grasses, dissolving the residue in 25% acetyl bromide in acetic acid and measuring by spectrometer the absorption at 280 nm.

This report established a correlation coefficient of -0.923 between IVD and the acetyl bromide method, whereas Van Soest found correlation coefficients between digestiblity and ADF, crude fibre, detergent lignin were -0.81, -0.75, -0.78 respectively.

2.2.2.2 In Vivo -

In vivo digestiblity involves housing the animals in metabolic crates, and keeping the animals on a maintenance level of feeding, collecting the faeces and determinating digestibility from equation (1). This result is only apparent, due to contribution from endogenous sources.

animal digestibility = Wt(feed)-Wt(faeces)/Wt(feed)*100

[equation (1)]

Development of rumen fistulation led to evolution of the nylon bag technique which involves suspension of samples inside a nylon bag in the rumen of an animal through the cannula. Chenost et al. (1970) found high correlations with in vivo values. However, others have reported less satisfactory results. The differences may be due to parameter values such as the aperture size of the bags, sample size in relation to the bag, duration of incubation, place in the rumen, and washing procedure.
Demarquilly and Chenost (1969) also included an acid-pepsin stage.

Samples in the nylon bag technique are in a dynamic environment in which fermentation products and bacteria are continously being removed from the rumen. When compared with in vitro, products accumulate throughout the fermentation in only 50 ml of fluid. Therefore VFA composition and digestion activity may differ from that of the rumen.

2.2.2.3 Pepsin-Cellulase -

The pepsin-cellulase assay has been developed to eliminate the dependence on rumen inoculum, thus removing a source of error between batches.
Donefer, Crampton and Lloyd (1973) found correlation between cellulase solubility and in vivo digestibility to be poor, and no better than solvent extraction.
Jarrige et al. (1970) however , have claimed better prediction than the two stage method of Tilley and Terry (1963). Jones and Hayward (1973) used a commercial preparation of cellulase from Trichoderma viride with cellulase, hemicellulase and proteolytic activity. The authors reported the method as rapid, reproducible and had a high correlation coefficient with in vivo digestibility.

Jones and Hayward (1975) reported a close correlation of pepsin plus cellulase solubility to in vitro digestibility $r = 0.93$, compared to $r = 0.96$ by Tilley and Terry (1963). Differences in the reports may be due to parameters, such as temperature and time, or differences in the activities of the enzyme preparation.

Treatment of herbage with acid- pepsin prior to incubation with cellulase resulted in improved correlation with both in vitro and in vivo digestibility. However this improvement was obtained only when pepsin treatment preceeded cellulase incubation. This suggested the pepsin removal of protein made the cell wall more accessible to subsequent cellulase digestion.

Goto and Minson (1977) used a modification of the Jones and Hayward method, containing 2.5% cellulase ONAZUKA SS (P-1500) extracted from T. viride and found the method valid for both temperate and tropical grasses. However Terry et al. (1978) found the method of Jones and Hayward (1975) was not sufficiently accurate to estimate in vivo digestibility for temperate legumes and grass -legume mixtures.

Mcleod and Minson (1978) in a study of the effects of particle size, sample size, incubation time and temperature, and cellulase concentration, found these parameters did not have a consistent effect on all the samples.

Samples for dry matter solubility (DMS) in the range of 40-60% had similar values when ground through different size screens, but for samples of 80% DMS, the DMS was 6% higher for finely ground samples.

T. viride cellulase has optimum activity at 50 °C and therefore increasing incubation temperature from 39 to 50 °C should result in higher DMS values. Rexen (1977) found no change in DMS for straw over the temperature range 35-50 °C nor did Jones and Hayward (1973) who found no significant increase in the amount digested from cocksfoot samples when incubation temperature was raised from 40 to 47 °C. This decreased when increased to 55 °C. However Mcleod and Minson (1978) reported a mean DMS increase of 4.4% , from 55.6 up to 60.0% after incubation for 48 hours at 39 °C and 50 °C respectively. The effect of incubation temperature was not consistent with all samples.

This is implied by the report of Terry et al (1978) with DMS response of legumes and grass differing. Effect of the incubation temperature on the DMS on the two classes is shown in table 2.2.2.3

Table 2.2.2.3

	Change in DMS	
	33 °C	50 °C
legumes	no effect	+3.5
grass	+2.5	+7.0

This suggests that DMS is not only a function of enzyme activity, but also of substrate class and their constituents.

T. viride cellulase is more active towards cellulose than hemicellulose. Therefore a substrate containing a greater proportion of of cellulose will show a more marked DMS response. Incubation time reduction lowered DMS as did reducing sample size. However when cellulase concentration was lowered from 2.5% (w/v) to 0.625% (w/v) no differences in mean DMS were found.

2.2.2.4 In Vitro Digestibility -

In vitro digestibility (IVD) has been found to be a good predictor of in vivo digestibility. However many variants of this method exist.(Quick et al., 1959; Hershberger et al., 1959; Donefer et al., 1960; Baumgardt et al., 1962; Tilley and Terry, 1963).

Barnes et al. (1964) carried out a comparison of in vitro fermentation methods; these being adaptations of the above methods. Differences were found between fermentation periods, and an interaction between periods and methods occurred, this being a result of changing rates of cellulose digestion between time periods. But this difference between methods disappeared after 18 hours.

A collaborative study involving 17 laboratories by Barnes (1967) showed that variability between laboratories was greater than among batches within laboratories, and this

was greater than between duplicate determinations within batches. The mean IVD for individual laboratories, after a 24 hour fermentation period ranged from 40.0 to 63.9% for cellulose and 38.7 to 53.3% for dry matter.

Digestibilty increased as the incubation period increased, but at different rates for grass and legumes, with precision being greatest for the longest fermentation periods.

The two stage technique of Tilley and Terry (1963) has become the most widely accepted for IVD. Although the method produces good correlation with in vivo values and reproducibility, there are inherent analytical errors between the two methods.

In Vivo digestibility is not constant, but varies with the species of ruminant used in the trial, age and health status, level of feed intake, and feed preparation (Whether feed is pelleted or chopped).

Because the larger variability of the in vivo, than the controlled conditions of in vitro , it is now considered preferable to report the results as in vitro. Ruminant digestion is a dynamic system in which there is a constant entry of digesta and removal of fermentation products. In contrast in vitro provides a stagnant environment in which protozoa and bacteria are not removed from the fermentation system, with the volatile fatty acids accumulating in the fermentation vessel.

In vitro digestibility therefore has a tendency to diverge from the ruminant situation. Relative measurements of in vitro digestibility have validity, due to good precision, but these should not be interpreted as absolute values. Some souces of undesirable variation are particle size, sample size, dilution ratio rumen fluid, inoculum pH and nitrogen avalability.

In vitro values were similar to *in vivo* values when animals were fed hay (hay inoculum), however the *in vitro* values were depressed with straw innoculum, but increased again when urea was added to the buffer. Therefore an inadequate supply of nitrogen will reduce the activity of the bacteria, although the animal will be less affected by this condition because of urea recycling by the saliva.

A pH range of 6.7 to 6.9 is similar to that of the rumen of sheep fed grass, although the range may be considerably greater than this depending on the diet. With a herbage containing a high content of soluble carbohydrate, the pH may be as low as 5.5 and up to 7.0 with poor quality hay.

Bacteria preferentially digest sugars and starches also resulting in a lower pH. This has been shown by supplementing the diet with a readily available carbohydrate leading to a reduction of the amount of fibre digested. (Raymond and Terry, 1966)

Knipfel and Troelson (1966) fed wethers with different combinations of: alfalfa hay, wheat straw and barley grain. IVD values decreased as donor diet deviated increasingly from IVD substrate. Results showed alfalfa possessed a growth promoting effect on microflora.

Digestibility differences were small among animals and less significant than between days. This may be due to various dilutions of inocula arising from consumption of water by animals prior to sampling for rumen fluid. (Troelsen and Hanel, 1966).

The work of Mcleod and Minson (1969) confirms that of Raymond and Terry (1966). Fastest rate of digestion was found to occur at pH 6.7, with longer duration of digestion reducing digestibility differences between pH's.

Particle size was found to have no effect on IVD by Tilley and Terry (1963), as did Mcleod (1972), who found finer grinding did not alter mean IVD and therefore did not improve accuracy of IVD for predicting in vivo DMD.

In contrast, the authors Minson and Milford (1967), Minson and Mcleod (1969), found finely ground samples led to a marked increase of mean IVD after 24 hours incubation. Rate of digestion was less for coarsely ground samples, especially the more mature forages. The magnitude of difference varied between species. Increasing the duration of incubation led to increased IVD and also reduced differences caused by grinder screen size.

Very fine grinding (ball milling) greatly increased IVD, with the most marked increase being samples of low digestibility initially.
(Virtanen, Pew, Dehority, and Johnson, 1963). This may be due to disruption of plant cell wall structure to allow enzymes to penetrate to areas from which they are normally excluded. It appears the effect of grinding will be influenced by the sample.

Sample size had no effect on IVD (Tilley and Terry, 1963; Mcleod and Minson, 1969; Mcleod, 1972). The mean effect of a larger sample size was to reduce the difference between duplicates, resulting in improved precision for the method. Conversely, reducing the samples size from 0.5 g to 0.1 g caused the residual standard deviation relating IVD to in vivo DMD to increase.

Dilution ratio of strained rumen fluid /buffer solution had a prominent effect on IVD (Mcleod and Minson, 1969). Increasing the proportion of strained rumen fluid increased the digestion rate. The ratios 2.5ml/47.5ml and 5/45 produced large deviations from the 10/40 recommended by Tilley and Terry. However 15/35 and 25/25 produced less significant deviations, and after 96 hours incubation, most samples showed the same IVD. As a measure of rumen fluid

activity, 65% innoculum IVD is suggested as the minimum for reliable data between batches (Brundage, 1972).

Studies of rumen temperature on fermentation showed it had no significant effect on digestibility, except for 0 °C water teatment, which required 108 minutes to attain initial rumen temperature (Brod et al. 1982). This data suggests that short periods of moderate temperature drop will not have any effect on microbiological activity.

Each of the parameters described above may be sources of IVD variation. However, rumen fluid will contribute the greatest error since it is the least controllable. Poor quality rumen fluid will produce the most significant deviation from the true value with samples of low digestibility.

IVD shows better correlation to in vivo digestibility than any of the chemical methods dicussed above. Although the pepsin-cellulase method is close to IVD as a predictor of in vivo digestibility, this precision does not hold for mixed species samples (grass + clover). Thus IVD is the desirable method for mixed herbage quality analysis even though it requires the inconvenience of an animal donor for rumen liquor.

2.3 GLC SEPARATION OF VFA

Free fatty acid analysis was first recognised by (James and Martin, 1952). Their study of C1-C12 acids showed that organic or inorganic acids added to the liquid phase was necessary to reduce tailing of the peaks as this reduces peak separation and causes peak area measurement errors.

Stearic and sebacic acid (Ackman and Sipos, 1964) were used initially to reduce tailing. Subsequently inorganic phosphoric acid was introduced to allow operation at higher temperatures. More recently however, introduction of formic acid vapour to the carrier gas has been shown to reduce

tailing at both moderate and elevated temperatures.

Double peak formation occurred with DC 550 silicone oil containing 5% stearic acid, Tween 80, and Neopentyl adipate (NPGA) (Ackman and Burgher, 1963). Any sample containing more than 1 ul water was likely to show double peak formation, the effect being most prominent with acetic acid. This was confirmed by Cochrane (1972) and in addition showed it occurred when injected just above the packing in a metal column, but this was absent in glass columns.

Water vapour sweeping through the column clearing adsorption sites and reactivating them, with subsequent adsortion of acid has been suggested as the process being responsible.

A dip below baseline (negative Peak) between solvent and acetic acid peak has been noted by several authors. Ackman and Sipos (1964) injected formic-water mixture, noted the dip occurred in the same position and concluded it was due to formic acid or some impurity in the formic acid which desensitized the detector. Ackman and Burgher (1963) also mentioned a dip.

However Di Gorcia and Samperi (1974) noticed the dip occurred after the propionic peak on Carbopack A with PEG 20M. This was attributed to water emerging from the column.

Free fatty acid separation had been carried out in 1952, and ghosting had been described by Erwin et al. (1961) Marco and Emery (1961), and Smith and Gosnell (1962). But it was Ackman and Burgher (1963) who developed a convenient method to eliminate the ghost peaks by continuous addition of formic acid vapour to the carrier gas.

Ghosting results from the reversible adsorption of a polar solute, with ghost peaks having identical retention times and shape to the solutes of the preceeding injection. The insidious nature of the problem is due to inability to recognise its occurrence and therefore its effect on

quantitative analysis.

Adsorption sites close to the injection area are implicated because of similarity between ghost and solute peaks. Glass wool column plugs and charred deposits from biological fluids are also indicated (Geddes and Gilmour, 1970) and this is in agreement with Ackman and Burgher (1963).

Because interaction between sample and support causes peaks to tail, the support structure is expected to affect peak width. Active sites on the support surface form hydrogen bonds with the sample, the strength of which determines the extent of tailing. VFA's have been noted as severe.

Reduction of tailing can be carried out in three ways (Supelco bulletin 723E)

1. remove active sites by acid or base washing
2. modification of surface by silanization
3. covering active sites by with stationary phase having polar functional groups.

Acid washing removes mineral impurities and extraneous materials while silanization is not suitable for aqueous samples. Polar functional groups such as ester, ether, hydroxyl or amine will have strong hydrogen bonding characteristics to tie up active sites. Analysis of VFA's requires the stationary phase to contain an acid to deactivate the support.

Formic acid was best for demonstrating ghost peaks as water was not always successful. With formic acid added to the sample it has been found ghosting still occurred. While a glass lined injection port system curtails the problem, formic acid in the carrier gas was essential to overcome ghosting (Van Eename et al. 1974)

Use of radioactive C14 labelled formic and acetic acids showed metal columns adsorbed 1.3 to 2.5% of the acetic acid. (Sokolowska, 1974)

Various methods have been tried unsuccessfully to eliminate ghosting. Among these, separately or in combination: alternate injections of water and sample; alternate injections of formic acid solution and sample; (reduces ghosting); use of inert teflon support (successful but low efficiency requiring a long column); coating the solid phase with 1-3% orthophosphoric acid (H_3PO_4). Unfortunately this was destructive to some stationary phases/supports. (Lemoine et al. 1965)

The most successful method was the introduction of a continuous stream of formic acid vapour into the carrier gas, although this was not completely successful for analysing a bacteriological medium. The severity of ghosting correlates with the amount of charred deposits built up in the injection port area. Although many authors have stated formic acid does not produce any response with flame ionisation detectors, Cochrane (1975) noted a standing current producing 15% full scale deflection on the recorder, due to acetic acid impurities in the formic acid.

It was concluded this did not pose a problem since it was possible to analyse free fatty acids (FFA) down to concentrations of 1 ppm. In a fundamental investigation into FID response to water and formic acid, Schafer (1975) found water produced both positive and negative responses, whereas pure formic gave no response under any condition. Water arriving in the carrier gas may lower the flame temperature and with it the sensitivity. Formic acid being the strongest of the mono-carboxylic acids, preferentially binds the active sites to supress the adsorption of the weaker acids.

A simple device for saturating chromatographic carrier gas with formic acid vapour is described by
Woo and Lindsay (1980), while
Duthie, Wulff, and Clark (1983), described a device which also includes a formic acid refill resovoir.

Component separation may be effected by a porous polymer (GSC) or a liquid phase coated on a solid support (GLC) An optimum support should have the following characteristics:

1. large specific surface area
2. uniform pore diameter of 10 micron or less
3. inertness
4. regular shape
5. mechanical strength.

Particle size is usually stated in terms of screen openings as follows:

MESH	MICRONS
60/80	250-177
80/100	177-149
100/120	149-125

60/80 mesh means the particles have passed through a 60 mesh screen but will not pass through an 80 mesh. Column efficiency improves with decreasing particle size, but back pressure usually increases. At present 80/100 is the mesh most commonly used. The effect of the support was demonstrated by Ramsey and Demigue 1974. Carbowax coated on Chromosorb P (AW) at 10% separated free fatty acids, but separation of C3/i-C4 was not possible on Chromosorb W.

Table 2.3A lists the packings evaluated, with the relevant authors and comments.

Table 2.3A

LIST OF PACKINGS EVALUATED, AUTHORS and COMMMENTS

Cabopack A/PEG 20M
Di Gorcia and Samperi (1974)
Separates 2-methylbutyric and 3-methylbutyric acids.
Suitable aqueous samples.

Chromosorb W/ Carbowax 20M/ H_3PO_4
Chromosorb W/ Carbowax 20M-TPA
Byars and Jordan (1964)
Carbowax 20M-TPA chemically crosslinked to make it more polar, tailing on both.

Chromosorb W/ 10% FFAP
Baker (1966)
FFAP is a reaction product between Carbowax 20M and 2-nitro terephthalic acid.Acetic acid mixtures not resolved.

Chromosorb W/ SP1200
Ottenstein and Bartley (1971A)
sharp peaks, no tailing, suitable aqueous samples.

Chromosorb T (Teflon 6)/ 10% Carbowax 400
Ottenstein and Bartley 1971B
Symmetrical baseline separation, but Chromosorb T has poor column efficiency.

Tween 80, DEGA/H_3PO_4, PEGS/H_3PO_4, Silicone DC50, have also been tried, but these are not suitable for acqueous samples.

Table 2.3B

POROUS POLYMERS RECENTLY EVALUATED, AUTHORS AND COMMENTS

Porapak Q 80/100 mesh, 4% H_3PO_4
Mahadevan and Stenroos (1967)
i-C4/C4 incompletely separated, valeric acid retention time 20 minutes.

Porapak Q 150/200 mesh, 4% H_3PO_4
Madahevan and Stenroos (1967)
i-C4/C4 incomplete separation, valeric acid r.t 45 minutes.

Porapak N
Ackman (1972)
Retention times very long. Assessed with formic vapour.

Chromosorb 101
Ackman (1972)
Acetic peak barely detectable without formic vapour.
Peaks have good symmetry with formic vapour (stainless steel columns)

Chromosorb 101
Ottenstein and Bartley (1971B)
Almost baseline separation of C1-C5 acids

Coating Chromosorb W (aw) with 6% FAL-M produced excellent separation initially, but deteriorated and it was not useable after 2 weeks (Thompson, 1984).

Differences between the results of Ackman (1972) and, Ottenstein and Bartley (1971B) for Chromosorb 101 are due to column preparation and operating parameters. Some of these factors are: temperature/time, whether conditioning was carried out with formic vapour; whether column was metal or glass; column length; packing mesh size; injection directly onto packing: column diameter; H_3PO_4 addition to sample; gas flow rate; injector , column and detector temperatures; and addition of H_3PO_4 to the liquid phase.

Ackman had used a stainless steel column when Ottenstein and Bartley had previously published a paper stating "In no case were H_3PO_4 modified stainless steel Chromosorb 101 columns as good as the Chromosorb 101 columns in glass. Neither Aluminium nor stainless steel columns could elute acids without formic acid."

Ottenstein and Bartley conditioned their Chromosorb 101 overnight at 250 °C in contrast to 165 °C overnight by Ackman. Ottenstein and Bartley also found that Porapak Q did not readily separate iso and n butyric acids nor iso and n-valeric acids.

Chromosorb 102 after reaction of the vinyl double bond with HF acid, has been shown by IR spectroscopy to be the source of adsorption (Hertl and Neumann, 1971). However this was not effective for acetic acid, but coating the support with Carbowwax 20M liquid phase improved the acetic acid peak. This is also applicable to Chrormosorb 101 since Chromosorb 102 differs only by the amount of cross-linking by divinylbenzene (Analab, 1979). Smith et al, (1978) show that bromination of the vinyl double bond for Chromosorb 102 resulted in a decrease of 60% adsorption associated with the vinyl groups, but this was not effective for carboxylic acids.

Although the papers by Mahadevan and Stenroos (1967), Ottenstein and Bartley (1971A, 1971B), and Ackman (1972), give comparative assessment qualitatively, no effort was

made to evaluate these differences quantitatively. Descriptions such as ghosting and tailing were given but precision and accuracy were not specified.

While Di Gorcia and Samperi (1974) mentioned "quantitative analysis with a precision good to within 3%", they did not mention the method of measurement which has a profound effect on the precision of the result. Each method has an inherent imprecision as follows:

Table 2.3C
Precision comparisons between methods of peak area measurement

	Time/trace minutes.	Precision
planimeter	45-60	4.06%
triangulation	45-60	4.06%
H*W*1/2	50-60	2.58%
Cut and weigh	100-200	1.74%
Disc	15-30	1.29%
Digital integr.	5-10	0.44%

Even with a digital integrator, reproducibility and precision is meaningless unless parameters for peak identification and integration are stated since tailing can lead to errors of peak recognition, this being due to integration termination when the slope drops below the value specifed in the instrument. Therefore peaks with long tails of gentle slope may lead to substantial errors.

In their study of column technology for the separation of aqueuos C2-C5 free acids, Ottenstein and Bartley (1971B) arrived at several conclusions:

1. glass columns produced the best results.
2. Modified terephthalic acid did not completely deactivate stainless steel tube.

3. silanized supports gave low response to acetic and propionic acids.

4. H_3PO_4 was most effective for deactivating support and stainless steel.

5. Teflon support produced good peak symmetry and resolution. But efficiency only about half that of diatomite support. Required longer column and retention times.

6. Metal at inlet was more detrimental than at outlet, producing erratic chromatograms. Suggested either glass lined injection port or on column injection.

7. Glass wool plug

   - untreated, caused considerable tailing
   - silanized, no peaks appeared
   - H_3PO_4, good peak symmetry

8. Low polarity phase gave better resolution for C3/i-C4 with decreased retention time but separation of n-C4/i-C4 became limiting.

Both temperature and phase polarity affect peak resolution. i-C4/C3 and i-C4/n-C4 are the most difficult pair of peaks to separate. The following table 2.3D shows separation factor decreases as temperature increases.

Table 2.3D

EFFECT OF TEMPERATURE ON SEPARATION FACTORS

column temp °C	125	150	175	200
separation factor i-C4/C3	1.37	1.27	1.19	1.14
separation factor n-C4/i-C4	1.26	1.21	1.16	1.12

column 6ft*2m glass U,10% SP1200/1% H_3PO_4,flow 40 ml/min

Table 2.3E : Comparison of liquid phase separation factors

phase	column temp.	separation factor i-C4/C3	separation factor n-C4/i-C4	McReynolds No. 2-methyl-2-pentanol
EGS	110	1.08	1.38	633
DEGA	110	1.12	1.37	479
EGA	110	1.14	1.37	462
FFAP	110	1.14	1.37	423
PEG 20M	110	1.18	1.36	387
SP 1200	110	1.37	1.26	145
POR Q	220	1.79	1.16	...

EGS = ethylene glycol succinate
DEGA = polydiethyleneglycol succinate
EGA = polyethylene glycol adipate
PEG 20M = polyethyleneglycol 20M
POR Q = Porapak Q

Abridged from Ottenstein and Bartley (1971)

As can be seen in the table, decrease of McReynolds number is accompanied by an increase of separation factor for propionic and iso-butyric acids, but decrease of separation factor for n-butyric and iso-butyric acids.

A critical factor in separation is the use of small samples. Large samples lead to peak broadening and tailing. This can be countered by reducing sample size, increasing inlet and detector temperatures. To obtain sharp peaks, the sample should be injected very quickly.

As reported in Smith and Waddington (1968), within a class of compounds there is a linear relationship between the retention time and the boiling point of the eluant. This was explained by Saura-Calixto et al. (1983) as a consequence of specific retention volume and the vapour pressure of a solute.

This means a plot of log retention time versus number of carbon atoms for an homologous series, is a straight line. Since Kovats retention index is related to the boiling point, this means boiling point within a homologous series can be of assistance in identifying unknown peaks.

Although many papers have been published on GLC separation of VFA's, no method with good precision for the quantitative separation of VFA's exists, because of adsorption of acetic and propionic acids by the support. Also present electronic integrator technology has progressed to the point where the lack of precision in columns has become very obvious. The Shimadzu data processor used also presented an opportunity to set up an internal standard method for quantitative measurement. Therefore a formic acid vapouriser for the GLC had to be constructed for addition of formic acid vapour to the carrier gas, and several GLC packings were evaluated for their suitability to separate VFA's quantitatively.

CHAPTER 3
EXPERIMENTAL METHODS

3.1 EXPERIMENTAL SITE

The plots were sown in 1977 and had been used for rotational grazing trials. Records for the sowing rates were not available. However these were likely to have been (Matua/white clover 20/3 kg/ha) and (Nui/white clover 15/3 kg/ha). Although not measured, there were large differences in plant densities between the cultivars. These were estimated to be 30 plants/m^2 for Matua and 150 plants/m^2 for Nui. The Matua/white clover and Nui/white clover swards did not receive any fertilizer nor irrigation during the collection period.

Matua prairie and Nui ryegrass were collected on adjacent areas of 100m by 100m in Mid-Canterbury. Each area was divided in the following manner:

2 mainplots = Nui, Matua
6 subplots = 6 replicates
10 sub-subplots = 10 date treatments

1. after the 2 mainplots were topped by a tractor mounted mower, each was divided into 12 one metre wide subplots.

2. 6 subplots were chosen at random in each mainplot and mowed to ground level with a rotary mower. These 6 replicate subplots had 0.5m borders also cut to soil level for buffer areas between subplots. This was carried out on 5 May 1981 and were assigned starting date A

3. The 6 subplots were then divided into 15 sub-subplots of 0.5m length. These were coded numerically from 1 to 15. At each 2 weekly sampling date, each sub-subplot was chosen from a random number table. The size of these sub-subplots were 1m * 0.33m.

Procedures 2 and 3 were repeated one week later on 12 May 1981 for starting date B. The sample collection dates for starting date A and starting date B are shown in table 3.1. Figure 3.1 shows the collection site plot layout.

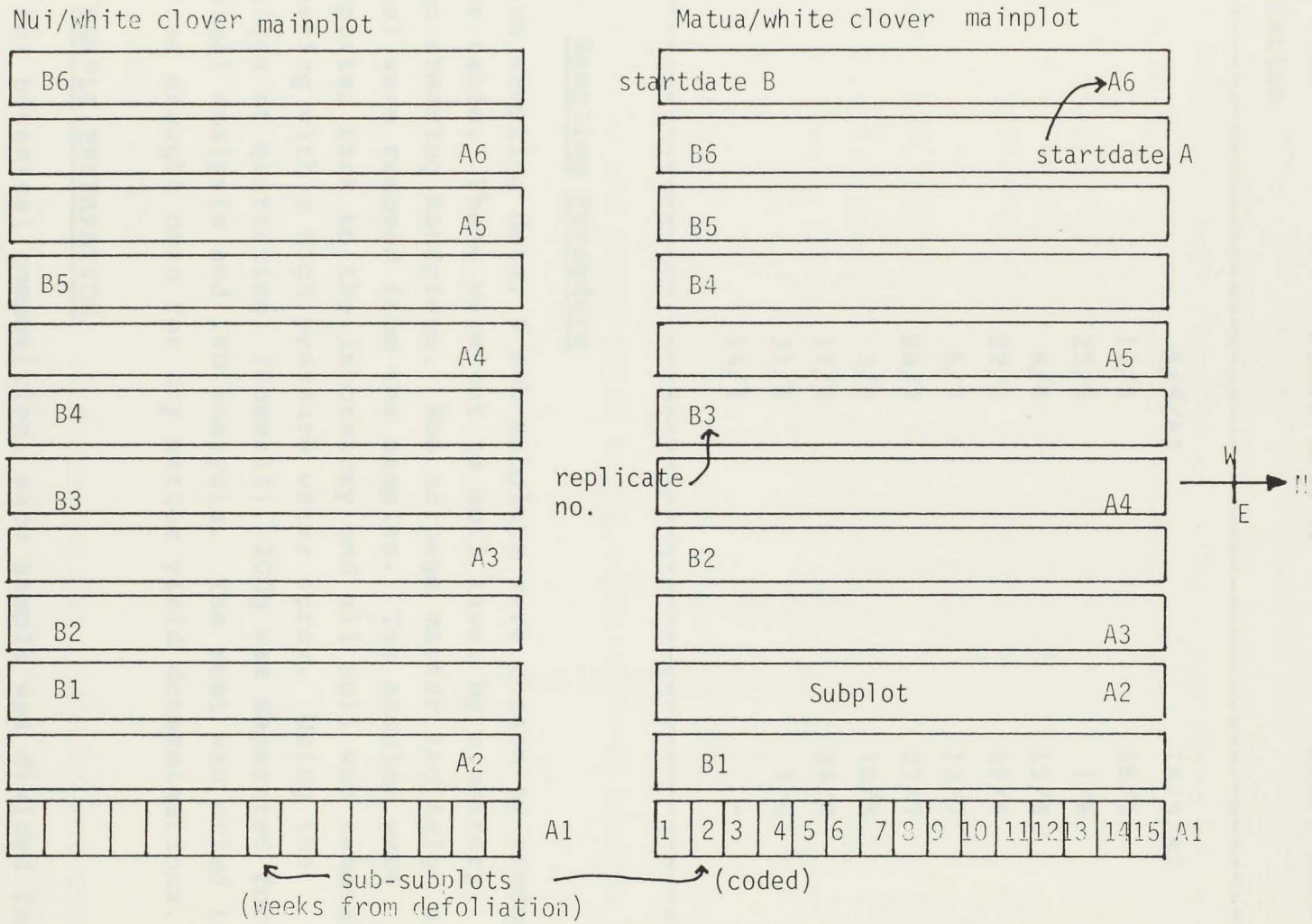


Figure 3.1: collection site plot layout

Table 3.1 : Collection dates for samples

Weeks from defoliation	Start date A	Start date B
0	5/5/81	18/5/81
2	12/5	18/5
4	25/5	1/6
6	8/6	15/6
8	22/6	29/6
10	6/7	13/7
12	20/7	27/7
14	3/8	10/8
16	17/8	24/8
18	31/8	7/9
20	14/9	-

3.1.1 Sampling Procedure

At each sampling date, 6 sub-subplots were chosen by random number table. These were cut to soil level by a battery driven shearing handpiece. Non herbage matter (eg twigs and stones) were removed from the samples. The samples were transported back to the laboratory and all soil was removed by washing with a high pressure water spray. Using the technique of quartering, (Boswell), 200g was separated for botanical analysis and IVD analysis. The rest was dried in a forced draught oven for dry matter yield determinations.

3.2 SAMPLE PREPARATION

For botanical composition, each sample was divided into grass, clover, weeds and dead matter. These were placed in paper bags and were dried to constant weight in a bench oven at 100 °C and weighed.

The samples for IVD analysis were spread thinly in aluminium trays with wire mesh bottoms. This allowed exposure of largest possible area to the air flow to enhance the drying rate. The samples were dried at 100 °C for 1 hour to deactivate enzymes which cause loss of soluble carbohydrates through respiration. The temperature was then lowered to 60 °C and dried to constant weight.

The samples were ground through a 1mm sieve in a Christy and Norris mill. The ground samples were then stored in sealed glass vials until analysis.

3.3 IN VITRO DIGESTIBILITY

The method used was the Moore modification of the Tilley and Terry (1963) two stage in vitro digestibility method.

The initial stage involves the microbial fermentation of soluble and structural carbohydrates to VFA end products, with proteins and amino acids being degraded into ammonia at this stage.

The protein escaping bacterial activity was then solubilized by the following acid pepsin incubation stage. At this stage, 6 ml of 20% HCl was also added to inhibit microbial activity. This simulates the process occuring in the abomasum of the ruminant.

The dry matter disappearance was then used to calculate the digestibilty of the sample.

3.3.1 Rumen Liquor

The greatest source of digestibility difference between batches occurs through variation of the rumen fluid. This may arise through changes of microbial population, or through dilution of rumen fluid from water intake by the animal prior to liqour collection. Variation in rumen liquor was minimised by:

1. placing the animal on a rationed diet with a strict feeding schedule.

2. and using a mixed fluid taken from two animals.

The animals were fed 1 kg good quality lucerne hay per day and the rumen fluid was always collected at 8.30 am on Monday following a 24 hour period in which no feed was given to the animals.

The fluid was collected by lifting the rumen until a sufficient quantity of the fluid had flowed out of the fistula into a 4 litre plastic pail. The fluid was strained through 4 layers of muslin cloth into a 1 litre thermos flask (prewarmed to 39 °C with water) until full to keep the fluid anaerobic. This was used to transport the fluid back to the laboratory.

3.3.2 Artificial Saliva

Prior to rumen fluid collection, a water bath operated by a time switch was set to preheat 3200 ml of distillled water to 39 °C. Preweighed chemicals in the ratios of table 3.3.2A were dissolved and gassed with oxygen free carbon dioxide until the solution reached pH 6.9.

Table 3.3.2A CHEMICALS FOR 3200 ml of ARTIFICIAL SALIVA

chemical	weight (g)
$NaHCO_3$	31.36
Na_2HPO_4	12.05
KCl	1.82
NaCl	1.50
$MgSO_4.7H_2O$	0.38
glucose	2.93
urea	2.93
$CaCl_2$ 4% w/v	3.2 ml

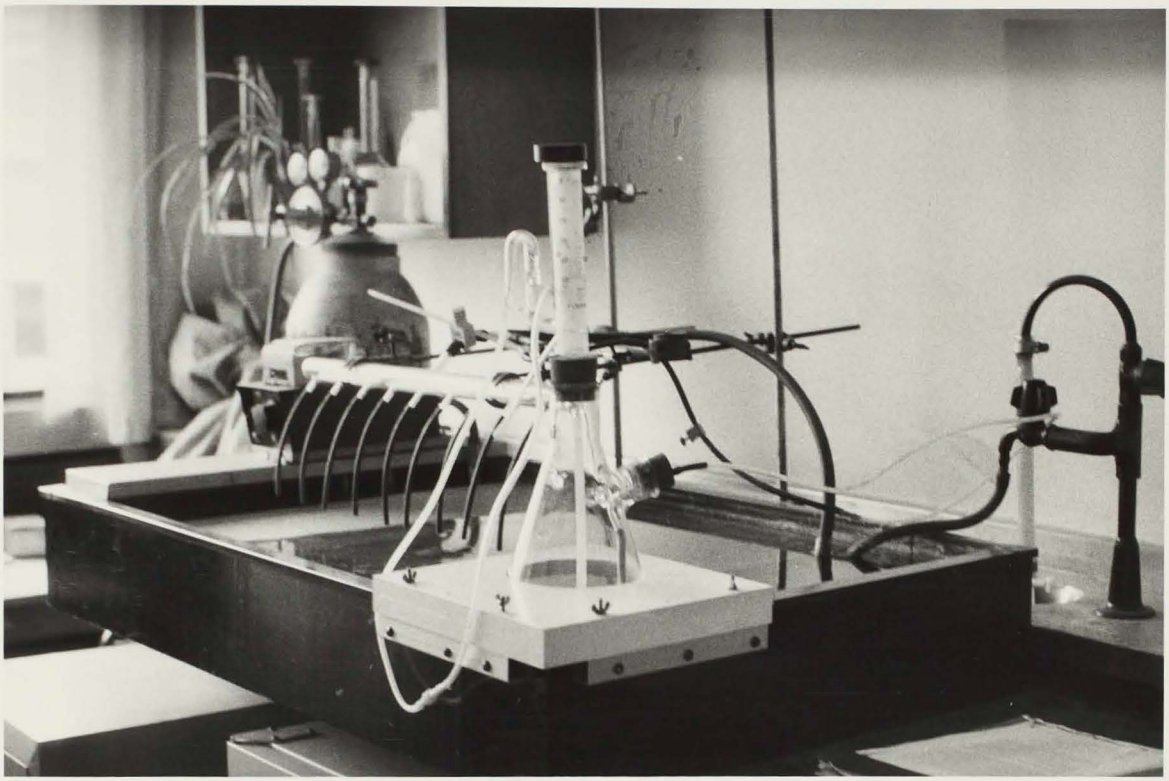
After collection of the rumen fluid, 800 ml was added to the artificial saliva and mixed thoroughly. A low flow of CO_2 was used to maintain an anaerobic atmosphere and a constant pH, while mixing the inoculant.

3.3.3 Bacterial Fermentation

Between 500 mg and 525 mg of sample was accurately weighed into 125 ml conical flasks. Three ml of distilled water was added to each sample to pre wet the samples to prevent floatation during addition of the inoculant. Each batch consisted of 60 samples, 4 blanks, 4 replicates of lucerne standard and 4 replicates of hay standard. Samples were tranferred to the water bath, and the air displaced with CO_2. The carbon dioxide was brought up to 30 °C by passing through a stainless steel heat exchanger immersed in the water bath.

An automatic pipettor (figure 3.3.3) was used to dispense exactly 50 ml of innoculant to each flask.

Figure 3.3.3 : Photograph of automatic dispenser



The samples were then fermented for 48 hours in a fan circulated, air incubator at 39 °C. A fan circulated incubator was necessary to remove the temperature differential between the upper and lower racks.

The incubator temperature can drop to below 25 °C for over 3 hours after transfer of sample from the water bath. This initial depression of temperature was solved through wiping the flask dry before transferring from the water bath to the incubator. It was caused by the vapourisation of water drawing off heat from the incubator.

The flasks were anaerobically sealed with rubber stoppers containing a pressure release valve, which allowed fermentation gases to escape to maintain a constant pressure, while preventing air from entering the fermentation vessel.

3.3.4 Acid-pepsin Digestion

1. a stock solution of 20% v/v HCl acid was made by adding 400 ml concentrated HCl acid to 1600 ml distilled water.

2. a fresh pepsin solution was made each week at a concentration of 5 g per 100 ml distilled water. This was prepared by weighing 7.5 g pepsin powder (1:10 000) (Sigma P-7000, activity 1750 units per mg protein) into a mixing cylinder and made to 150 ml with water.

These were then combined by mixing together in the ratio of 150 ml pepsin solution plus 450 ml of 20% v/v HCl acid solution.

An automatic dispenser was used to pipette 8 ml of the HCl-pepsin solution into each sample. Foaming occurred due to production of gas from buffer neutralization of HCl.

This was caused by lipids and protein material raising the surface tension of the fluid and trapping the released gas.

By addition in small aliqouts and shaking to allow the gas to escape, the problem was considerably reduced. The sample was then incubated for a further 48 hours at 39 °C, mixing three times daily. Anaerobic conditions were not necessary at this stage.

3.3.5 Filtration

Following acid-pepsin digestion, the contents of each flask were filtered through preweighed gooch crucibles (porosity 1) It was necessary to weigh the crucibles for each batch since the crucibles may lose up to 10 mg of the glass sinter from furnacing. The filtrate was drawn off under vacuum and any material remaining in the flask was loosened with a rubber covered glass rod and rinsed into the crucible with hot distilled water.

During filtration, lipids and small particles which entered the glass sinter caused it to become blocked. Use of celite 545 filter aid, and pouring off supernatant fluid before rinsing out solids, reduced this problem considerably.

The crucibles were transferred to a 105 °C oven, and the residue dried for 24 hours. The samples were transferred directly into a dessicator to cool. They were weighed out of the dessicator as the residue can absorb up to 10 mg of moisture from the atmosphere in 1 hour.

The crucibles were furnacedat 500°C for 3 hours to remove all traces of organic material before re-use.

3.3.6 Dry Weight

Analysis errors due to residual moisture can arise from two sources:

1. samples dried to constant weight at 60 °C during sample preparation while IVD residue is dried at 105 °C

2. stored samples may absorb moisture from the atmosphere if not stored in sealed containers (up to 10% of the sample weight).

These errors were eliminated by carrying out separate sample dry weight% determinations at 105 °C as follows:

C = (dry wt / initial wt) * 100

This can then be used to correct the sample for residual moisture:

dry sample wt = (initial wt sample * C)/100

3.3.7 Calculations

$$IVD\% = \left[1 - \frac{\text{residue wt} - \text{blank wt}}{\text{dry sample wt}} \right] * 100$$

Residue is the undigested fraction of the sample, and is therefore the complement of in vitro digestibility. A blank consisting of inoculant without sample was taken through the same procedure. This blank gives the weight contribution by the solid material which passed through the muslin when the rumen fluid was strained.

3.3.8 Determination Of Animal Digestibility For Batch Standards

This was carried out to determine the apparent animal digestibility for both the meadow hay and lucerne hay used as batch references. A preliminary 7 days were used to adjust the sheep to the diets, and by the weight of the feed refused, an average daily intake of 800g for the animals was established.

The trial was then carried out in the following manner with six animals:

1. 400 g feed at 8.30 am
2. 400 g feed at 4.00 pm
3. faeces collected at 8.30 am, dried in a forced draught oven for 24 hours, then weighed.
4. Percent dry weight of the feed was determined by drying to a constant weight in a forced draught oven, then weighed. The dry weight of the feed was then calculated as follows:
5. feed dry wt = 800g * (% dry wt of the feed) /100
6. in vivo digestibility was then calculated using feed dry weight.

$$\text{in vivo digestibility} = \frac{(\text{feed dry wt} - \text{faeces dry wt}) * 100}{\text{feed dry wt}}$$

3.4 VFA ANALYSIS

3.4.1 VFA Sample Preparation

This was adapted from Supelco bulletin 749E proceedure A. At the end of 48 hours the fermentation fluid was mixed, and centrifuged at 1500 rpm for 5 minutes to sediment the sample.

Four ml of the fluid was withdrawn with a 5.00 ml pipette (accurate to 0.01 ml) and ejected into 1.00 ml of internal standard solution. This solution contained 25% orthophosphoric acid (H_3PO_4) for deproteinisation, and crotonic acid (trans-2-butenoic acid, 50 mg per 100 ml) as internal standard. (Crotonic acid is not found in rumen fermentation products.)

The 30 ml capped polypropylene tube was inverted several times to mix the sample thoroughly, and allowed to stand for 30 minutes. It was then centrifuged at 2000 rpm for 10 minutes in an MSE benchtop centrifuge to sediment solids, and stored below 4 °C. At this temperature fermentation ceases. Phosphoric acid precipitates protein thus preventing enzyme activity allowing the samples to be stored for long periods prior to analyses.

The VFA samples were analysed on a Varian 2800 GLC. Separation was carried out on a 2400mm by 3mm i.d. glass column filled with Tenax GC/6% FAL-M (80/100 mesh). The GLC had a formic acid vapouriser fitted to saturate the carrier gas with vapour just prior to entering the column. See figure 3.4 and diagram 3.4

Figure 3.4A : Photograph of formic acid vapouriser

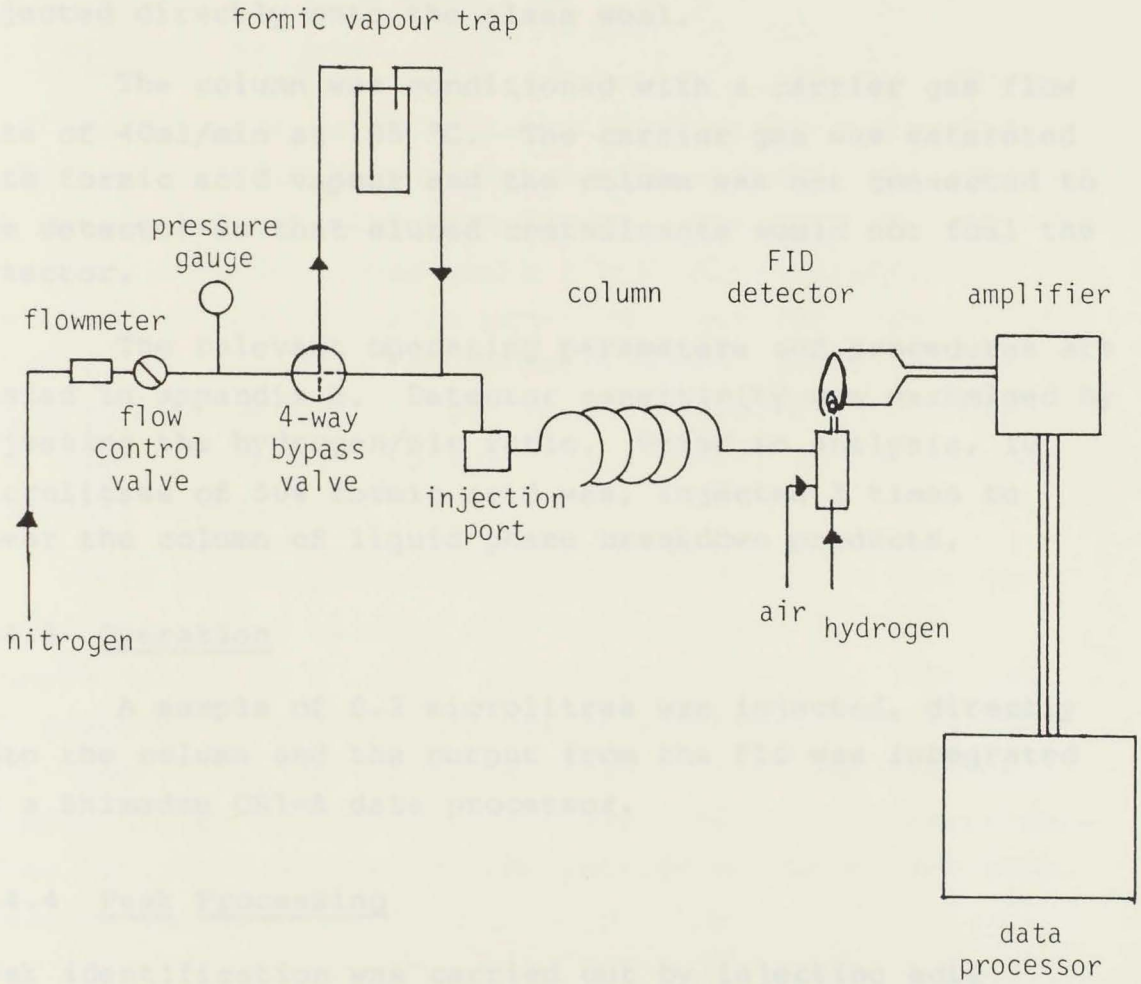


DIAGRAM 3.4 : Outline of Formic Vapouriser Connections.

3.4.2 Column Preparation

The column was soaked in chromic acid overnight to oxidise any organic material, rinsed and then soaked in 4% orthophosphoric acid overnight to deactivate any hydroxyl sites on the glass surface. The column was thoroughly rinsed with distilled water, packed with Tenax GC/6% FAL-M and plugged at both ends with glass wool washed in 4% H_3PO_4. The front of the column was packed so that the sample was injected directly onto the glass wool.

The column was conditioned with a carrier gas flow rate of 40ml/min at 185 °C. The carrier gas was saturated with formic acid vapour and the column was not connected to the detector so that eluted contaminants would not foul the detector.

The relevant operating parameters and procedures are listed in appendix B. Detector sensitivity was maximised by adjusting the hydrogen/air ratio. Prior to analysis, 10 microlitres of 50% formic acid was, injected 3 times to clear the column of liquid phase breakdown products.

3.4.3 Operation

A sample of 0.2 microlitres was injected, directly onto the column and the output from the FID was integrated by a Shimadzu CR1-A data processor.

3.4.4 Peak Processing

Peak identification was carried out by injecting acid standards and determining their retention times. These were entered into the ID table in the integrator memory and used to calibrate the response factors, using the one point calibration method (see section 4.5.3 and section 4.5.4, chapter 4). Individual VFA concentrations were then calculated by the data processor using peak areas and response factors.

CHAPTER 4
RESULTS

4.1 ANALYSIS OF DATA

The data was analysed using the Genstat statistical package. Analysis of variance of the variates were carried out by a 3 factor split-plot design. The factors were cultivar, startdate, and weeks (from defoliation). Treatment of the data also provided tests for interaction between the factors. The variates were: yield, grass%, clover%, dead matter%, IVD%, nutritive value index, total VFA, acetic/propionic molar% ratio, molar% acetic and molar% propionic acids.

Where normalization of percentage distributions were necessary, paired missing data were omitted before transformation by 57.297 * arcsin(sqrt(%/100)). The data was smoothed over both axes before regression was carried out. Regression curves are plotted on the same axes to examine trends and to make relative comparison between data sets. Standard errors for the regression curves are also drawn in the plots.

4.2 YIELDS

Yields (kg/ha) of dry matter of swards differed significantly between cultivars (table 4.2A) and was higher for Nui. Although Matua reduced this difference with time, it did not reach the same level as Nui. Nui had more rapid tillering and Matua is disadvantaged by defoliation down to soil level. Yield difference between starting dates was also significant. A starting date one week later increased

mean yield of dry matter. Interaction between cultivar and weeks from defoliation show that the Nui yeild increasingly diverged from Matua as regrowth proceeded. The start date for Nui showed the initial yields were different up to week 8, but converged after this (figure 4.2A and table 4.2A)

4.2.1 Sward Composition

Differences in grass% were caused by cultivar, starting date, and interval from defoliation. There were interactions between cultivar*startdate, cultivar*weeks, cultivar*startdate*weeks. The time from defoliation also affected the relative differences between the cultivars. This is illustrated in figure 4.2B and table 4.2B. The Nui grass% (31.4%) was much higher than Matua grass% (3.8%) at week 2, but this difference converged to 65.1% and 64.1% respectively by week 20 ie. (cultivar*weeks). Nui grass had a rapid initial regrowth reaching a plateau by week 14 while Matua grass as a proportion of the sward changed only slowly, but increased rapidly from week 14. The cultivar*startdate significance is illustrated by the 4 distinct curves for the startdates A and B, for each cultivar.

The Nui white clover sward proportion had a mean difference of 22.4% higher than Matua over the collection period. This may be attributed to the clover density being substantially reduced through shading by the Matua plants. Differences of clover% between swards were 24.6% at week 2, 29.2% at week 6 (greatest difference) and 22.6% at week 20. There was no cultivar*weeks interaction, the changes in proportion showed similar trends in both swards (figure 4.2C and table 4.2C). During sample collection, Matua sward clover plants were observed to be much larger and taller, but the Nui sward clover were more dense.

Dead matter differed significantly between the cultivars in the Matua and Nui sward samples, and this was affected by the starting dates (table 4.2D). The pecentage dead matter decreased with increase of time interval from defoliation. Interaction between cultivar*weeks occurred. For Nui samples, this decreased much more rapidly than Matua and had a narrower range. Interaction between startdate*weeks showed startdate B had a higher dead matter% for the first 10 weeks after defoliation. It converged to similar values as Nui dead matter% in the last 10 weeks.

Figure 4.2A : DRY MATTER YIELD BETWEEN VARIETIES : COLLECTION PERIODS A & B

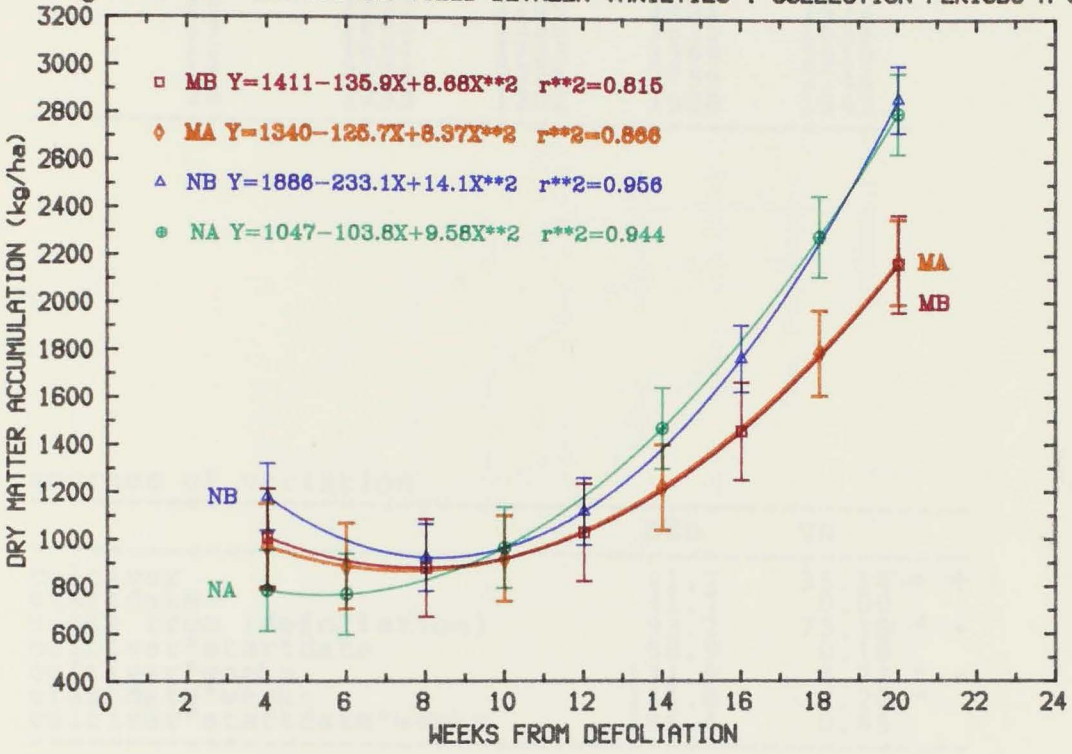


Table 4.2A: mean yield (kg/ha) dry matter

weeks	Matua A	Matua B	Nui A	Nui B
2	956	964	1227	1244
4	745	958	704	1097
6	1103	775	956	751
8	1003	1260	965	954
10	723	944	1056	1110
12	1233	1104	1651	1472
14	1602	1228	1635	1629
16	1684	1723	1368	2510
18	2174	2326	2759	2744
20	1233	1202	1508	1542

sources of variation

	SED	VR	
cultivar	41.7	35.14	* +
startdate	41.7	0.00	
weeks from (defoliation)	93.2	75.18	* +
cultivar*startdate	58.9	0.10	
cultivar*weeks	131.8	4.72	* +
startdate*weeks	131.8	2.20	*
cultivar*startdate*weeks	186.4	0.66	
S.E.	322.8		

* significant P(<.05)
\+ " P(<.01)

Figure 4.2B: EFFECT OF DEFOLIATION ON GRASS REGROWTH

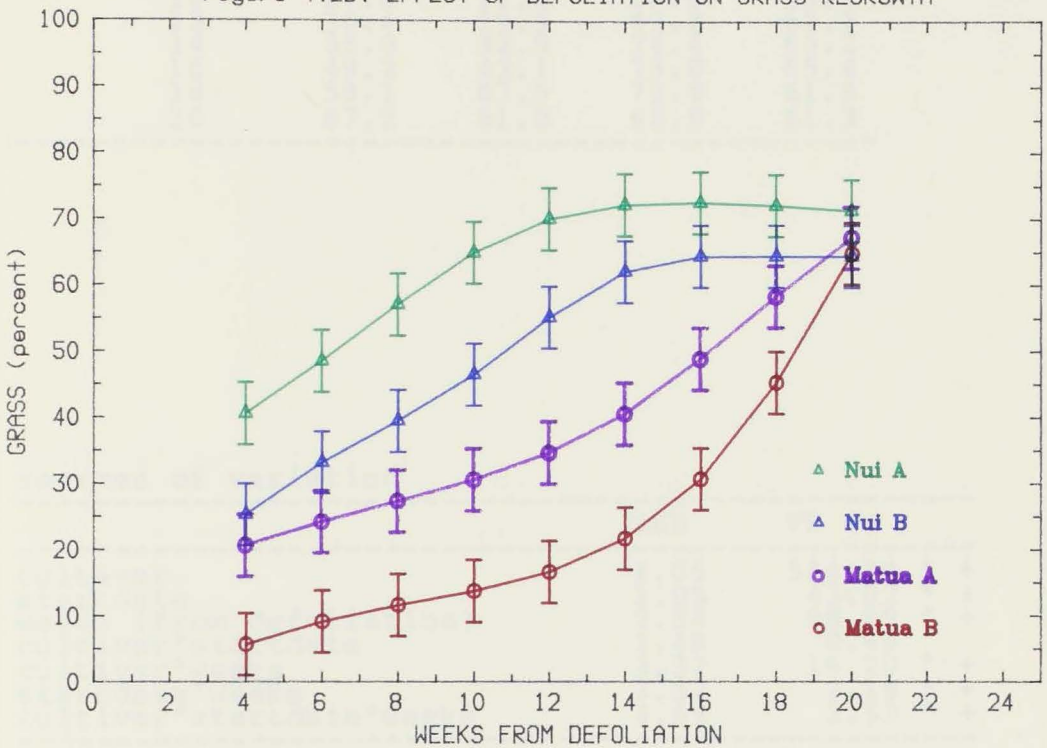


Figure 4.2C: CLOVER GROWTH PATTERN AFTER DEFOLIATION

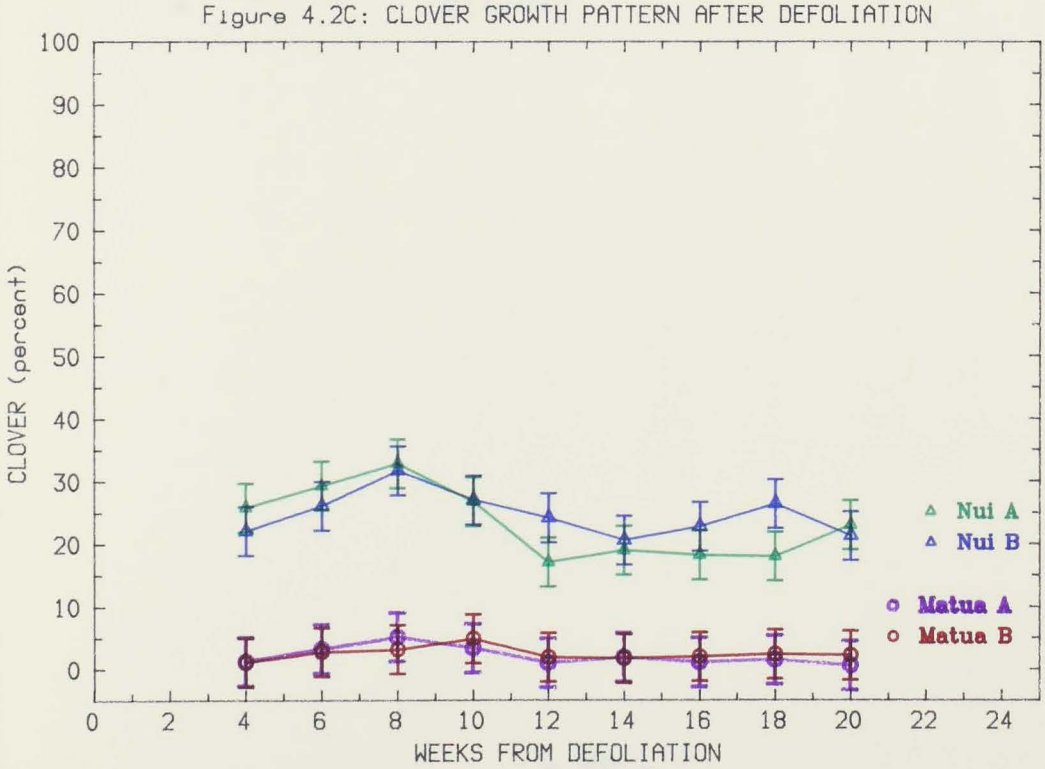


Table 4.2B: mean grass%

weeks	Matua A	Matua B	Nui A	Nui B
2	2.8	4.7	33.4	29.3
4	20.8	11.1	40.5	43.3
6	18.8	12.6	39.8	32.2
8	24.5	21.7	55.6	41.9
10	27.6	11.2	68.8	56.3
12	36.4	19.3	69.2	62.7
14	35.8	32.9	74.4	65.2
16	49.2	42.1	73.0	65.4
18	59.1	67.5	73.9	61.9
20	67.2	61.0	68.9	61.3

sources of variation

	SED	VR	
cultivar	1.05	534.34	* +
startdate	1.05	43.02	* +
weeks (from defoliation)	2.34	98.89	* +
cultivar*startdate	1.48	0.48	
cultivar*weeks	3.32	16.20	* +
startdate*weeks	3.32	2.19	* +
cultivar*startdate*weeks	4.69	2.53	* +
S.E.	8.13		

* significant P(<.05)
\+ " P(<.01)

Table 4.2C: mean clover%

weeks	Matua A	Matua B	Nui A	Nui B
2	2.8	0.7	30.7	21.9
4	1.2	1.5	25.8	26.0
6	2.3	2.1	31.4	31.5
8	5.1	4.7	32.8	26.9
10	3.3	1.6	26.7	23.9
12	0.8	1.5	17.0	20.4
14	1.9	1.8	19.0	22.5
16	1.0	2.1	18.1	26.1
18	1.4	2.0	18.0	21.0
20	0.5	0.3	22.9	23.0

sources for variation

	SED	VR	
cultivar	0.89	634.80	* +
startdate	0.89	0.00	
weeks (from defoliation)	1.98	3.23	* +
cultivar*startdate	1.26	0.04	
cultivar*weeks	2.81	1.74	
startdate*weeks	2.81	1.37	
cultivar*startdate*weeks	3.97	0.69	
S.E.	6.87		

* significant P(<.05)
\+ " P(<.01)

Table 4 2D: mean dead matter%

weeks	Matua A	Matua B	Nui A	Nui B
2	91.8	93.3	26.9	48.8
4	78.7	85.9	29.1	27.9
6	77.2	83.4	26.8	33.4
8	60.1	71.6	4.7	26.9
10	62.8	81.0	0.0	15.6
12	56.2	64.7	7.7	11.1
14	53.1	56.6	2.9	7.5
16	44.4	36.5	3.4	5.6
18	29.3	16.4	2.1	4.2
20	29.7	35.8	4.9	11.4

sources of variation

	SED	VR	
cultivar	1.06	1844.44	* +
startdate	1.06	35.96	* +
weeks (from defoliation)	2.37	103.17	* +
cultivar*startdate	1.50	0.00	
cultivar*weeks	3.35	17.86	* +
startdate*weeks	3.35	6.39	* +
cultivar*startdate*weeks	4.73	2.97	* +
SE	8.19		

* significant P(<.05)
\+ " P(<.01)

4.3 DIGESTIBILITY

4.3.1 Animal Trial

Digestibilities for the grass hay and lucerne hay used as the batch references were determined by an animal feeding trial using 6 wethers. This resulted in the values:

grass	hay	63.2%
lucerne	hay	71.5%

4.3.2 Rumen Fluid

Because rumen fluid can be an unwanted source of IVD variation, measurements of the rumen pH were carried out at 2 hourly intervals for 24 hours after feeding. This established that pH declined (more acid) as fermentation produced VFA's from the digesta. This reached a minimum after 8 hours and rose again as the VFA's were absorbed from the rumen.

Likely batch variation of the rumen fluid was determined by measuring the pH of rumen fluid taken from the sheep. The sheep were fed 1 kg of lucerne hay daily on a regular schedule and starved for 24 hours before collection of rumen fluid. The results were mean pH 7.25, S.E. 0.24. The pH of the artificial saliva inoculant made with these rumen fliud could easily be reduced to ph 6.8-6.9 with CO_2 the level which produces the greatest rate of digestion.

4.3.3 In Vitro Digestibility

Analysis of variance of IVD between batches for the standard samples of hay and lucerne showed the difference between batches was not significant $P(<.05)$ and therefore IVD values were not adjusted between batches (see appendix D). Also the analysis of variance showed that the difference between the means of hay and lucerne did not change significantly ($P<0.05$) between batches (no interaction).

The variations of blanks between batches were in the range of 5 mg to 30 mg. Within batch variation of blanks had a range of 10 mg even though the rumen liqour had been filtered through 4 thicknesses of muslin cloth. To reduce the blank weight variation and to maintain a uniform pH for the initial point of fermentation, carbon dioxide was continuously passed through gas diffusers at a low rate into the innoculant to mix the fluid.

Foaming caused loss of sample during addition of hydrochloric acid-pepsin mixture due to some of the sample being carried out of the fermentation vessel by the entrapped gas. This was due to surface tension of the liquid trapping CO_2 released from neutralization of the buffer. Octan-2-ol was used to reduce the foaming (MAF Research Station, Winchmore).

To test its effect on the IVD, digestibilities of the hay standard were measured, and the samples without octanol were used as the control. There were 4 replicates of the control and 4 replicates of the octanol treatment. Analysis of variance showed that the addition of octanol did not alter the digestibility $P(<.05)$.

IVD was higher for the Nui samples (grass + clover + dead matter) throughout the collection period (table 4.3.3A). The cultivar, startdate,weeks from defoliation were all significant factors affecting IVD%. Also there were interaction between cultivar*startdate, cultivar*weeks, startdate*weeks, cultivar*startdate*weeks. The mean difference between the cultivars was 31.4%, and for start dates 3.3%. The lowest IVD were: Matua 14.8% and Nui 38.0% both occurring at week 6. The highest were 69.5% and 74.4% respectively. The start date had a greater effect on the Matua samples, reducing their mean IVD from 42.1% down to 36.8%. For Nui the reduction was 61.5% to 60.3%. Greatest difference between the start dates occurred at week 10 and this was a 10 unit reduction. (see table 4.3.3A)

The nutritive value index measures not only the digestibility, but also the dry matter available for digestion. Nui samples had higher values throughout the collection period. (table 4.3.3B). Significant factors were cultivar, and weeks from defoliation. The interaction cultivar*weeks was also significant.

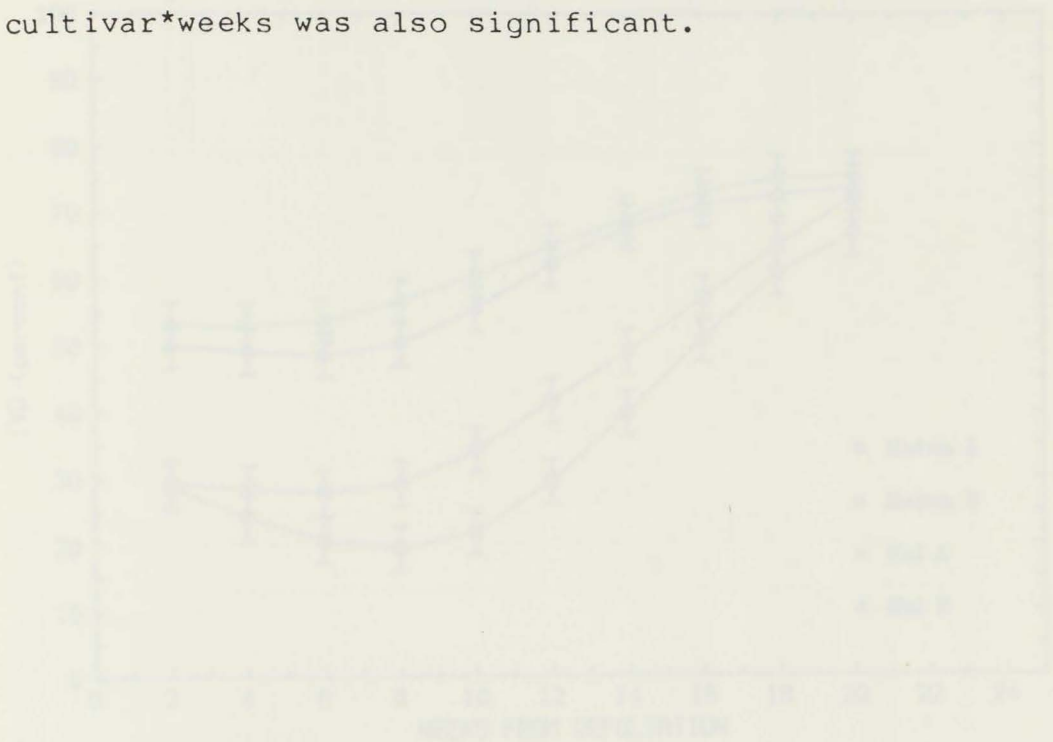


Figure 4.3.3: IN VITRO DIGESTIBILITY FROM GROWTH ACCUMULATION

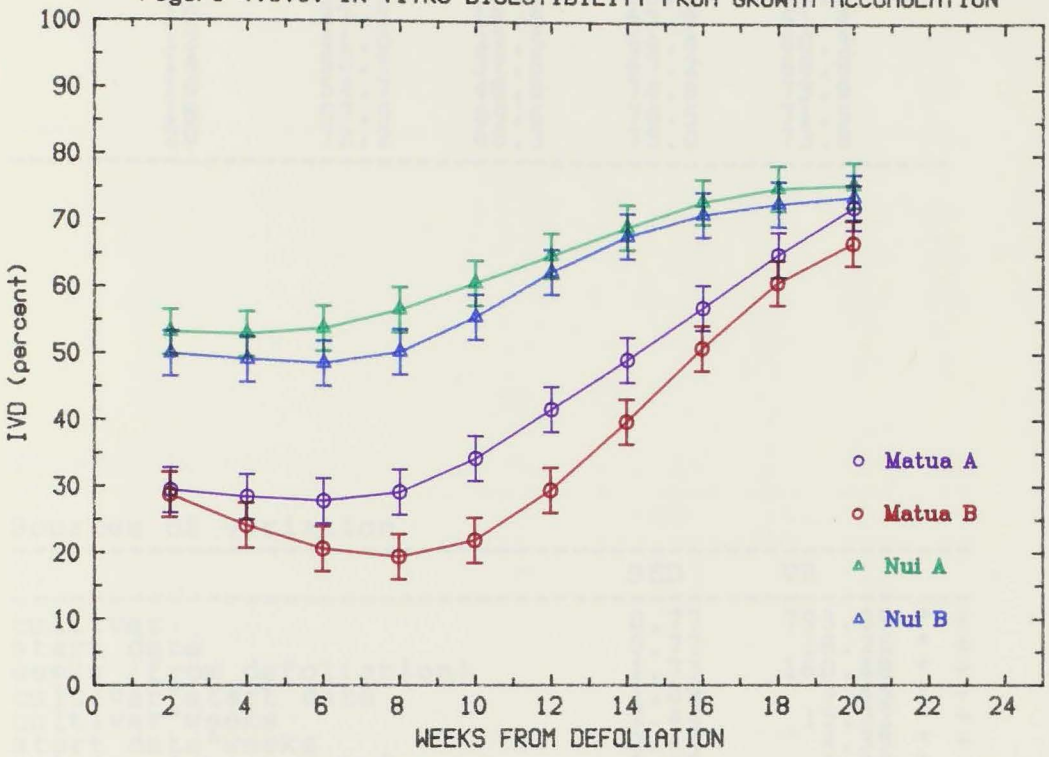


Table 4.3.3A: mean IVD% for samples containing (grass+clover+dead matter)

weeks	Matua A	Matua B	Nui A	Nui B
2	30.4	26.9	53.2	49.9
4	29.2	22.2	52.9	53.6
6	13.2	16.4	32.0	44.0
8	25.3	18.8	56.1	45.9
10	37.2	18.5	62.9	61.4
12	41.0	37.5	64.4	60.3
14	50.1	37.5	67.2	68.8
16	54.7	48.8	74.8	73.9
18	67.9	69.6	76.5	71.5
20	72.2	66.3	75.0	73.8

Sources of variation

	SED	VR	
cultivar	0.77	793.09	* +
start date	0.77	18.26	* +
weeks (from defoliation)	1.73	160.48	* +
cultivar*start date	1.09	7.45	* +
cultivar*weeks	2.44	15.22	* +
start date*weeks	2.44	4.39	* +
cultivar*start date*weeks	3.45	2.80	*
S.E.	5.98		

* significant P(<.05)
\+ " P(<.01)

Table 4.3.3B: mean nutritive value index

weeks	Matua A	Matua B	Nui A	Nui B
2	341	261	586	589
4	208	243	377	587
6	145	128	310	367
8	261	248	537	464
10	277	177	654	714
12	505	417	1065	902
14	810	483	1191	1148
16	1044	784	1786	1937
18	1484	1615	2114	1967
20	569	490	1967	958

sources of variation

	SED	VR	
cultivar	26.4	273.05	* +
startdate	26.4	1.98	
weeks	59.0	162.71	* +
cultivar*startdate	37.3	2.62	
cultivar*weeks	83.4	7.65	* +
startdate*weeks	83.4	1.23	
cultivar*startdate*weeks	117.9	1.73	
S.E.	204.3		

* significant P(<.05)
\+ " P(<.01)

4.4 FERMENTATION VOLATILE FATTY ACIDS

4.4.1 Storage Of VFA's

To determine the stability of VFA's to storage, fermentations were quantitatively separated on the GLC, then stored in a refrigerator at 4 °C. After 2 weeks these samples were re-analysed.

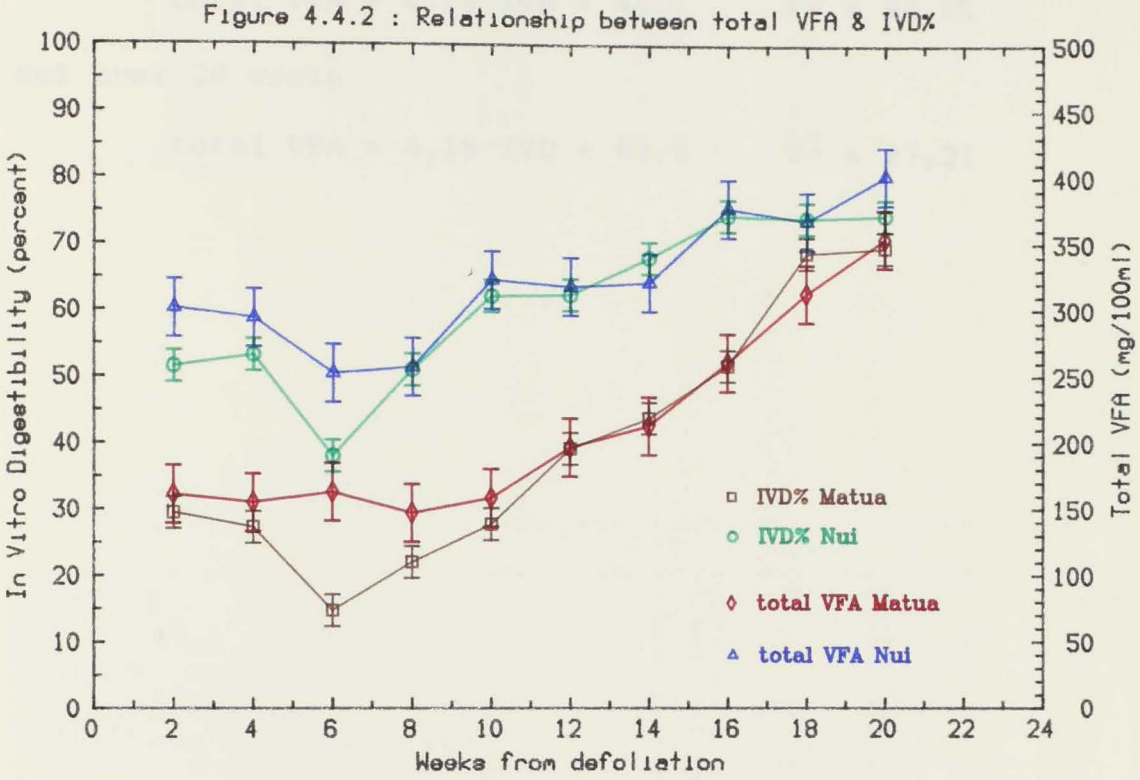
The separations did not show any difference for individual acids nor total acids. It was noted however, that after a longer period of storage, the samples became more viscous due to solids diffusing back into the liquid.

4.4.2 Acetic And Propionic Acids

The trend of acetic and propionic acid amounts (mmoles) were consistent with IVD and yield of dry matter. There were minimums at week 6, then a steady rise to week 20. The changes between acetic and propionic acids were inversely related, but the propionic acid only partially made up for the decrease in the acetic acid (mmoles). The minor VFA's iso-butyric, butyric, iso-valeric and valeric acids also increased. The acetic/propionic molar% ratios were significantly different for weeks from defoliation and there was also a cultivar*startdate interaction (table 4.4B). The amounts (mmoles) were sigificantly higher between cultivars for both acetic and propionic acids throughout the collection period for Nui.

The total VFA was different between cultivars and also for weeks after defoliation. These two factors were also the major source of variation for IVD%. To test for linkage between these two variates, regression of total VFA on IVD% for the two factors were carried out. The curves IVD% vs weeks, and total VFA vs weeks have been plotted on the same axes in figure 4.4.2.

Figure 4.4.2 : Relationship between total VFA & IVD%



The regression equations are:

Matua over 20 weeks

$$\text{total VFA} = 4.24\ \text{IVD} + 44.1 \qquad r^2 = 98.5\%$$

Nui over 20 weeks

$$\text{total VFA} = 4.18\ \text{IVD} + 63.6 \qquad r^2 = 87.2\%$$

Table 4.4A: total VFA (mg/100ml)

weeks	Matua A	Matua B	Nui A	Nui B
2	133	189	262	341
4	122	188	258	329
6	115	211	213	292
8	144	151	239	274
10	196	122	285	360
12	253	142	282	352
14	257	170	325	317
16	276	246	376	378
18	310	316	387	348
20	359	350	381	421

sources of variation

	SED	VR	
cultivar	6.88	248.29	* +
startdate	6.88	5.59	*
weeks (from defoliation)	15.39	30.00	* +
cultivar*startdate	9.73	12.33	* +
cultivar*weeks	21.77	2.87	* +
startdate*weeks	21.77	4.80	* +
cultivar*startdate*weeks	30.78	2.97	* +
S.E.	53.32		

* significant P(<.05)
\+ " P(<.01)

Table 4.4B: acetic/propionic acid molar% ratio

weeks	Matua A	Matua B	Nui A	Nui B
2	2.78	1.96	2.79	2.67
4	2.70	2.31	2.18	2.65
6	3.05	2.87	2.43	2.89
8	2.68	2.81	2.53	2.92
10	2.36	2.03	2.61	2.75
12	2.41	1.96	2.13	2.11
14	2.27	2.49	2.41	2.22
16	2.03	2.37	1.95	2.19
18	2.47	2.20	2.16	2.25
20	1.90	1.71	2.18	2.34

sources of variation

	SED	VR	
cultivar	0.079	0.39	
startdate	0.079	0.03	
weeks (from defoliation)	0.177	4.20	* +
cultivar*startdate	0.112	5.04	*
cultivar*weeks	0.249	1.21	
startdate*weeks	0.249	0.93	
cultivar*startdate*weeks	0.353	0.74	
S.E.	0.612		

* significant P(<.05)
\+ " P(<.01)

Table 4.4C: molar% acetic & propionic acids

acid	cultivar	startdate	week 2	week 18
acetic	Matua	A	65.4	57.0
		B	62.2	58.5
	Nui	A	63.8	59.7
		B	63.0	59.3
prop	Matua	A	24.1	31.6
		B	24.0	27.0
	Nui	A	26.1	28.8
		B	23.5	27.2

standard errors of differences of means

	acetic SED	prop SED
cultivar	0.304	0.258
startdate	0.304	0.258
weeks	0.304	0.258
cultivar*startdate	0.403	0.365
cultivar*weeks	0.430	0.365
startdate*weeks	0.430	0.365
Cultivar*startdate*weeks	0.608	0.516
S.E.	1.053	0.894

* significant P(<.05)
\+ " P(<.01)

sources of variation

	acetic VR	prop VR
cultivar	4.89 *	1.17
startdate	6.21 *	72.07 * +
weeks	268.94 * +	271.79 * +
cultivar*startdate	0.14	0.17
cultivar*weeks	12.66 * +	15.47 * +
startdate*weeks	17.26 * +	12.23 * +
cultivar*startdate*weeks	12.40 * +	27.98 * +

* significant P(<.05)
\+ " P(<.01)

4.5 EVALUATION OF GLC PACKING MATERIALS

All evaluations were carried out using a standard VFA sample made from analar chemicals, and varying the carrier gas flow rate, oven, injector and detector temperatures to find the opitmum separation for packing material.

4.5.1 Chromosorb 101 (80/100 Mesh)

This is a porous styrene divinyl benzene polymer. Because of the excellent results reported by Ottenstein and Bartley (1971), this material was tried initially. It had fast elution times, excellent separation, produced narrow peaks with excellent symmetry, with no baseline drift, and almost total absence of baseline noise. Tailing for both acetic and propionic acids were barely discernible.

Column conditioning was carried out for 3 days at 250 °C, and vibrated down as large cracks appeared due to shrinkage of the resin.

Operating the column at 205 °C and 40 ml per minute flow rate for nitrogen carrier gas, separation of VFA's up to to 5 carbon chain length valeric acid was possible in 5.5 minutes, with 6 carbon caproic acid (n-hexanoic) being separated in 8.8 minutes.

2-methylbutyric and 3-methylbutyric (iso-valeric) acids had similar peak heights. These could be separated, although only to about 50% peak height.

Two serious problems occurred with C101 packing material.

1. degradation of the packing was found to occur after 3 days. This was due to analysis of samples which had been prepared with orthophosphoric acid (for deproteinisation) turned the packing material a grey colour on the inlet side and concurrent deterioration of column performance.

2. lack of reproducibility due to adsorption by the packing occurred and could not be eliminated. After about 10 injections, acetic acid deviated up to 16% from the true value, and propionic acid to a lesser extent.

Introduction of formic acid vapour to the carrier gas could reduce this variation to only 12%. For this reason, C101 was abandoned for the analyses, and various other packings tried.

4.5.2 Other GLC Packings

Porapak Q (80/100 mesh). This which produced broad peaks with a retention time of 45 minutes for valeric acid. This was due to the large specific surface area of 600 square metres per gram compared to 50 for C101.

Chromosorb W (aw)/10% Carbowax-TPA + 0.5% MPA. This produced only 6 peaks from the standard sample containing 7 VFA's. By injecting acetic, propionic, iso-butyric, butyric, iso-valeric, crotonic and valeric acids individually, the retention times showed propionic and iso-butyric acids to be 3.4 and 3.7 minutes respectively. These two appeared as a single composite peak. Reducing the temperature and the carrier gas flow to improve resolution increased the total analysis time to 25 minutes, but could only separate the 2 acids down to about 80% peak height of iso-butyric acid. (ie 80% height of iso-butyric remained unresolved)

Chromsorb W (aw/DMCS)/5% OV 101 (80/100 mesh). One large peak appeared at 0.8 minutes, and a small peak at 3.3 minutes, the peaks being so erratic that measurement was not possible.

Chromosorb W/ FFAP Acetic, isobutyric, and butyric acids were not completely resolved, even after 17.6 minutes for valeric acid. Substantial tailing occurred for butyric

acid.

Chromosorb W (aw) / FAL-M 25%, DMCS treated. This produced erratic peaks due to water removing the DMCS

4.5.3 Tenax GC /6% FAL-M (80/100 Mesh)

Separation of VFA's could be achieved after conditioning the column overnight at 165 °C, at a carrier gas flow rate of 20 ml/ min.

However the reproducibility was 6% c.v. for acetic acid improving to 1.2% if 5 ul of 50% v/v formic acid was injected prior to analysis. The acetic acid c.v. steadily improved until a minimum of 0.5% was reached after one week, suggesting that column conditioning at a higher temperature and a longer period was desirable. A second column conditoned at 185 °C with formic acid for 3 days resulted in a more stable column.

This column was superior to C101 in baseline stability and peak symmetry, but elution of valeric acid was about 2 minutes longer. As with C101, a solvent peak appeared at he beginning of the chromatogram followed by a negative peak just before acetic acid appeared, but this did not interfere with the peak area integration.

Substituting 95% formic acid solution for formic acid (AR) in the vapouriser, almost eliminated this baseline dip from the Tenax column. However at a formic acid concentration of 80% v/v, almost no formic acid would vapourise. 100% formic acid (AR) was consumed at a rate of about 2 ml every 24 hours, whereas at 80% v/v, the level in the resevoir remained almost constant. Addition of formic acid vapour (AR) into the carrier gas produced a 20% increase of baseline level on the integrator.

With use, tailing of the peaks became gradually more evident. This problem could be controlled by removing the carbon deposits at the head of the column and replacing the

phosphoric acid washed glass wool plug. This packing material was used for all VFA analyses.

Due to the long retention time of 26 minutes for caproic acid, crotonic acid (unsaturated trans-2-butenoic acid) was substituted as the internal satndard. Its peak eluted between iso-valeric and valeric acids. This reduced elution time to 11 minutes, but required conditioning for a longer period at a higher temperature. Careful packing of the column to remove any irregularities and gaps was necessary to achieve the required separation. Table 4.5.3A to table 4.5.3F show the data processor parameters used, retention times for the analyses, response factors, output for the standard sample, the ID table for multiple calculation, and the conversion factors for the ID table. Figure 4.5.3A is an example of the output from a standard sample and figure 4.5.3B shows an output from in vitro lucerne hay.

4.5.4 Identification Of An Unreported VFA

All VFA separations contained a peak which has not been reported in previous publications on rumen VFA analyses. To identify this peak, the regression line of boiling point versus retention time for the acids, indicated a b.p. of 177 °C (Saura-Calixto, 1983). This considerably reduce the field of possibilities of the products from anaerobic fermentation. Two likely possibilities were 2,3-butanediol and 2-methylbutyric acid.

Because the polarity of the liquid phase affects the relative positions of the elutions, the above compounds were eluted on Tenax GC /FAL-M, and also on Porpak Q and Chromosorb W (aw)/Carbowax 20M-TPA. The area of the unknown peak in the fermentations were of similar size to iso-valeric acid (3-methylbutyric acid).

Table 4.5.3 A: Data Processor Parameters

CAL PM	1
WIDTH	5
SLOPE	235.7
DRIFT	0
MIN AR	1000
T-DBL	0
LOCK	1.8
STP TM	12
ATTEN	5
SPEED	4
METHOD	43
SPL WT	4
IS WT	2

C-R1A	
SMPL #	00
FILE #	1
REPT #	257
METHOD	43
CLB C1	1

Table 4.5.3 B: retention times

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.06			285984
3	PROP	3.56			240816
4	I-BUT	4.91		V	48337
5	BUT	6.1			117261
6	I-VAL	8.26			52679
1	CROT	9.76		V	119803
7	VAL	11.06		V	48747
	TOTAL		0		913631

ID TBL	1
MAX	7
MODE	1

Table 4.5.3 C: response factors

#	NAME	TIME	WINDOW	F1/F2	C1/C2
1	CROT	9.67	5	1	50
2	ACET	2.05		2.095	250
3	PROP	3.54		1.244	125
4	I-BUT	4.86		0.9914	20
5	BUT	6.05		1.022	50
6	I-VAL	8.16		0.9097	20
7	VAL	10.94		0.983	20

C-R1A
SMPL # 00
FILE # 1
REPT # 258
METHOD 43

Table 4.5.3 D: output for standard

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.06	249.9968		285984
3	PROP	3.56	124.9997		240816
4	I-BUT	4.91	19.9998	V	48337
5	BUT	6.1	49.9998		117261
6	I-VAL	8.26	19.9999		52679
1	CROT	9.76		V	119803
7	VAL	11.06	19.9997	V	48747
	TOTAL		484.996		913631

ID TBL 2
MAX 7
MODE 1

Table 4.5.3 E: (optional) ID table for multiple calculation

#	NAME	TIME	WINDOW	F1/F2	C1/C2 (mmol/l)
1	CROT	11.54	5	0	0
2	ACET	2.76		0.1665	0
3	PROP	4.52		0.135	0
4	I-BUT	6.01		0.1135	0
5	BUT	7.36		0.1135	0
6	I-VAL	9.69		0.0979	0
7	VAL	12.74		0.0979	0

Table 4.5.3 F:

*F1 = 10/MW This is a conversion factor to convert the result from mg/100 ml to M moles/litre for multiple calculation.

Acid	1 mg/100 ml	2 mg/1000 ml	3 MW	4 m moles/ℓ	5 ID TBL 1 C1	6 ID TBL 2 F1*
Crot	50	500	86.09	5.808	50	0.1162
Acet	250	2500	60.05	41.632	250	0.1665
Pro	125	1250	74.08	16.874	125	0.1350
I-but	20	200	88.11	2.270	20	0.1135
But	50	500	88.11	5.675	50	0.1135
I-val	20	200	102.14	1.958	20	0.0979
Val	20	200	102.14	1.958	20	0.0979
I-cap	20	200	116.16	1.722	20	0.0861
Cap	50	500	116.16	4.304	50	0.0861

			bp°C	Density	Solubility in water
Crotonic	trans-2-butenoic	$CH_3CH{:}CHCO_2H$	185	1.018	v
Acetic	Ethanoic	CH_3CO_2H	117.9	1.0492	∞
Propionic	Propanoic	$CH_3CH_2CO_2H$	140.99	0.9930	∞
Iso-butyric	2 methyl propanoic	$(CH_3)_2CHCO_2H$	153.2	0.96815	v
Butyric	Butanoic	$CH_3(CH_2)_2CO_2H$	163.53	0.9577	∞
Iso-valeric	3 methyl butanoic	$(CH_3)_2CHCH_2CO_2H$	176.7	0.9286	S
Valeric	Pentanoic	$CH_3(CH_2)_3CO_2H$	186.05	0.9391	S
Iso-caproic	4 methyl pentanoic	$(CH_3)_2CH(CH_2)_2CO_2H$	205	0.9225	δ
Caproic	n-hexanoic	$CH_3(CH_2)_4CO_2H$	205		i

v = Very soluble S = Soluble δ = Slightly i = Insoluble ∞ = Completely soluble.

SLOPE 188.6

START 03.30.13.41.

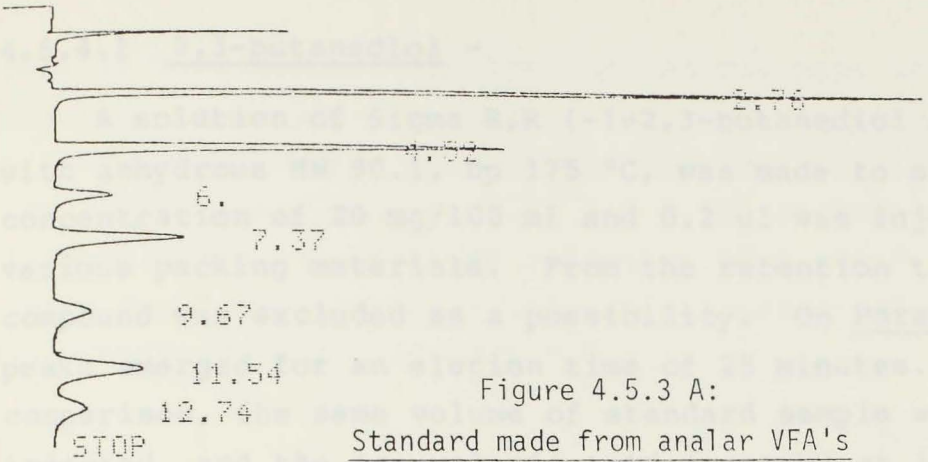


Figure 4.5.3 A:
Standard made from analar VFA's

C-R1A
SMPL # 00
FILE # 1
REPT # 111
METHOD 43

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.76	251.3988		108490
3	PROP	4.52	125.7894		92960
4	I-BUT	6.	20.0186	V	18415
5	BUT	7.37	49.4843		45335
6	I-VAL	9.67	20.1642		20459
1	CROT	11.54			45975
7	VAL	12.74	20.1802	V	20583
	TOTAL		487.0357		352219

C-R1A
START 03.30.17.58.

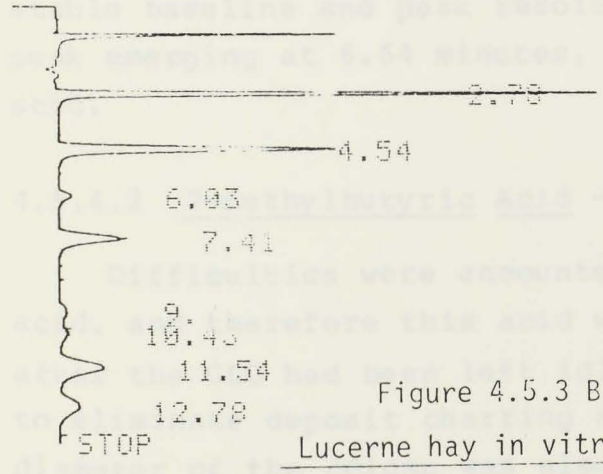


Figure 4.5.3 B:
Lucerne hay in vitro VFA's

C-R1A
SMPL # 00
FILE # 1
REPT # 112
METHOD 43

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.78	289.0617		64700
3	PROP	4.54	147.6909		56610
4	I-BUT	6.03	9.1163		4349
5	BUT	7.41	46.5242		22107
6	I-VAL	9.71	8.3545		4396
		10.43		V	4778
1	CROT	11.54		V	23846
7	VAL	12.78	25.8004	V	13648
	TOTAL		526.5483		194439

4.5.4.1 2,3-butanediol -

A solution of Sigma R,R (-)-2,3-butanediol AR with anhydrous MW 90.1, bp 175 °C, was made to a concentration of 20 mg/100 ml and 0.2 ul was injected onto various packing materials. From the retention time, this compound was excluded as a possibility. On Porapak Q, no peaks emerged for an elution time of 25 minutes. For comparison, the same volume of standard sample was also injected, and the iso-valeric acid appeared at 11.47 minutes, and crotonic acid eluted at 19.2 minutes as the last peak. A rumen fluid sample which had not been prepared with crotonic acid internal standard, contained only 6 peaks. It was therefore concluded that 2-3-butanediol does not elute on Porapak Q.

When injected alone onto Chromosorb W (aw)/12% Carbowax 20M-TPA, 2,3 butanediol appeared at 6.17 minutes. For the standard sample the propionic acid and iso-butyric acid emerge as one peak at 3.28 minutes, followed by butyric (4.58 min) , iso-valeric (5.32 min), valeric (7.17 min) and crotonic (8.57 min). A rumen fluid sample shows a less stable baseline and peak resolution, with the unidentified peak emerging at 6.64 minutes, just in front of valeric acid.

4.5.4.2 2-methylbutyric Acid -

Difficulties were encountered obtaining 2-methylbutyric acid, and therefore this acid was tested several months after the GLC had been left idle. The column was repacked to eliminate deposit charring at the inlet. The internal diameter of the column was also reduced from 3 mm i.d. to 2 mm i.d. to improve resolution. because the column had a narrower bore, the elution times changed. To overcome this problem, the relative retention time of the 2-methylbutyric acid to the iso-valeric acid was used to compare the results of the 2mm i.d to the 3mm i.d. column. The injection of rumen fluid on the 3mm i.d. column eluted the unidentified

peak at 9.71 minutes and the iso-valeric peak at 10.43 minutes. A standard sample containing 10mg/100ml s(+)-2-methylbutyric acid (Sigma Chemical Co., anhydrous MW 102.1) produced peaks at 7.24 and 7.79 minutes respectively. In both cases the relative retention time was 0.93, thus verifying 2-methylbutyric acid as the previously unidentified peak. (figure 4.5.4.2).

START 09.08.16.14.

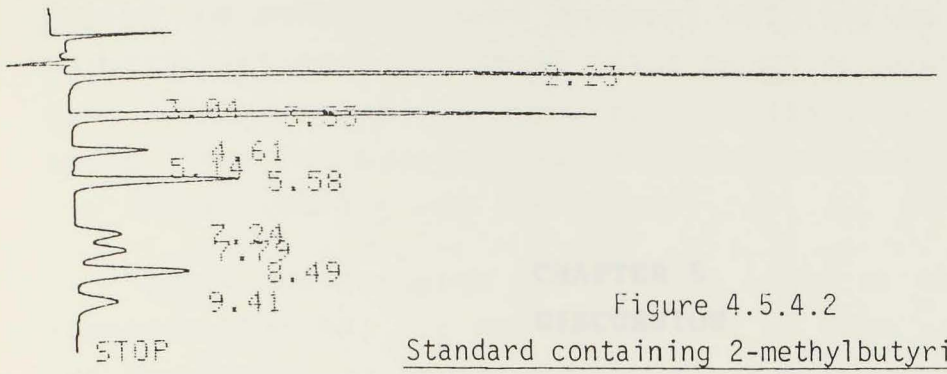


Figure 4.5.4.2

Standard containing 2-methylbutyric acid

C-R1A
SMPL # 00
FILE # 6
REPT # 775
METHOD 43

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.23	251.5761		89371
3	PROP	3.53	121.5856	V	74338
4	I-BUT	4.61	19.587		16038
5	BUT	5.58	50.2239		37237
6	I-VAL	7.24	21.3363		17048
		7.79		V	20696
1	CROT	8.49		V	37868
7	VAL	9.41	19.7939	V	16605
	TOTAL		484.1031		309204

CHAPTER 5
DISCUSSION

5.1 YIELDS

Production of dry matter is dependent on a number of factors, some of these being, sowing rate, accumulation, fertilizer, irrigation, soil composition, climate, cutting height, cutting frequency, and cultivar.

The results show that when defoliated to soil level in May, Nui swards recovered much faster than Matua swards for the period from the beginning of May to mid September. This is reflected in the narrower range of dry matter production and grass component of the sward for Nui. Nui sward dry matter yield was higher than Matua at both the beginning and at the end of the collection period (see table 4.2A). This may be explained by the differences in the growth characteristics between the cultivars. Matua plants had a low tillering rate. The lack of leaf area for initial photosynthesis reduced the plants capacity for regrowth. In contrast Nui increased its tiller density rapidly.

Various authors have carried out cutting trials with these two cultivars, and results reported here are comparable to their reports, when differences in trial conditions are taken into account (Rys et al., 1977; Baars and Cranston, 1977; Harris et al., 1977; Lancashire, 1978; Boswell, 1977).

Rys et al. (1977) in a trial at Flock House, Bulls, cut the sward to 3 cm when the ryegrass reached 700-800 kg/ha dry matter. The dry matter yield was 19% higher than Nui

ryegrass annually with most of the increase contributed during the summmer. Less frequent defoliation (28-46 days between cuttings) increased Matua sward production by 17% , with a Matua grass only component of 38% from January to April. For Nui however, sward production was 14% lower and the clover content was 48% lower.

Baars and Cranston (1977) in a trial at the Wairakei research station, cut the sward to 3 cm when lucerne was at 10% flowering, or at 15 cm height. Matua winter production was higher than Nui, with Matua superior from midsummer through to early spring, and Nui better from spring to early summer. Annual production was not significantly different between the cultivars.

Harris et al. (1977) cut the sward to 2.5 or 5.0 cm with a 3 month interval over winter. Mean values of yield were:

winter	1080 kg/ha
spring	5700
summer	1205
autumn	390

A herbage yield of 2919 to 3415 kg/ha for Nui over the winter was reported by Lancashire et al., (1978). Boswell (1977) reported cutting to a height of 3 cm at 2 weekly intervals out yielded 6 cm cuts in a predominantly ryegrass sward. (6630 kg/ha and 5340 kg/ha dry matter respectively)

5.1.1 Sward Composition

Matua swards produced very little green material for the first 4 weeks (15% grass) and growth was very slow until spring. Nui however recovered to about 42% grass 4 weeks after mowing, confirming the conclusion of Baars and Cranston (above) that less severe defoliation favoured Matua regrowth.

From week 18 onwards, the live material composition of the Nui sward began a slow decline, the amount of senescent material making a compensatory increase. This occurred for both starting dates and is apparent in the IVD figures.

In contrast, Matua sward live material continued on a steady increase as did the IVD. This pattern is contrary to that reported by Baars and Cranston (1977), who found higher winter yield for Matua swards. This can be explained by the initial severe defoliation of mowing to soil level in comparison to their cutting height of 3 cm. Plant growth accelerated at the beginning of September, most noticeably for the Matua plots. The white clover in these plots became much taller and larger in comparison to the Nui white clover. This was because the plant density was much less for both the grass and clover than in the Nui plots.

Clover proportion for the Nui swards was also higher throughout the period. The pecentage clover accumulation rose and fell in the same pattern in both the cultivar swards, suggesting the decline was due to grass competition. The cultivar had no interaction with the clover growth.

In a comprehensive paper on the growth characteristics of perennial ryegrass, Hunt and Field (1978) reported that the rate of appearance of leaves, root axes, and tillers increased rapidly for light irradiance up to 100 w/m^2 and for temperatures up to 20 °C. In swards where the tiller density is sufficient to induce tiller competition, differences in tiller density tend to be compensated by tiller growth. Tillering remained at a low level after grazing, then a short period of rapid tillering occurred. A tillering flush also occurred after defoliation, which explains the commonly observed higher tiller populations in frequently cut swards. Another determinant of tiller density is the tiller death rate, which increases regardless of density if the swards are undefoliated for long periods, and thereafter declines as a stable population is reached. Tillering remained at a low rate for 10 days after grazing

before a short period of rapid tillering. Tillering densities peaked in late winter-early spring.

The rapid recovery of leaves and tillers 2 weeks after defoliation; leaf growth; the appearance of senescent material; and the declining live material proportion in the sward at the beginning of September follows the pattern reported by Hunt and Field.

Due to the upright growth characteristics of Matua, dead matter contained much more debris from the structural material of the mature stem.

5.2 IN VITRO DIGESTIBILITY

The two stage in vitro technique described by Tilley and Terry (1963) has been reported by many authors to be accurate and highly correlated to in vivo digestibility.

Because of difficulty separating sufficient sward components for analysis, IVD was carried out on the bulked samples. This was composed of (grass+clover+dead matter). This description will apply to all following references to Matua, Nui, or samples.

The higher IVD for Nui samples throughout the collection period in comparison to Matua were due to a greater percentage of live matter, partly from grass, and also from higher clover proportion.

From the beginning of spring, IVD of Matua samples increase rapidly and almost equalled that of the Nui samples, corresponding to the increased rate of dry matter production and declining dead matter proportion. The Nui IVD reached a maximum at week 18 and then marginally declined, concurrent with the increase of dead and senescent material. Digestibility of the Matua samples however, continued to increase.

The regression of IVD against live matter (figure 5.2), shows that Nui samples had a much narrower range than Matua, demonstrating its rapid pasture regrowth after defoliation. The regression curve is a concave quadratic, and this shows that the IVD rose more rapidly than live matter percentage. This may be due to the secondary factor of decreasing digestible portion of the dead and senescent material from the time of mowing (as the soluble carbohydrate present was lost) concurrent with the increase of digestible live organic matter content.

Variations for IVD were quite large for some replicates, and this was especially noticeable for the initial samples of Matua. This was caused by the higher dead matter proportion, and the small amount of dry matter available from the low plant density in the subplots.

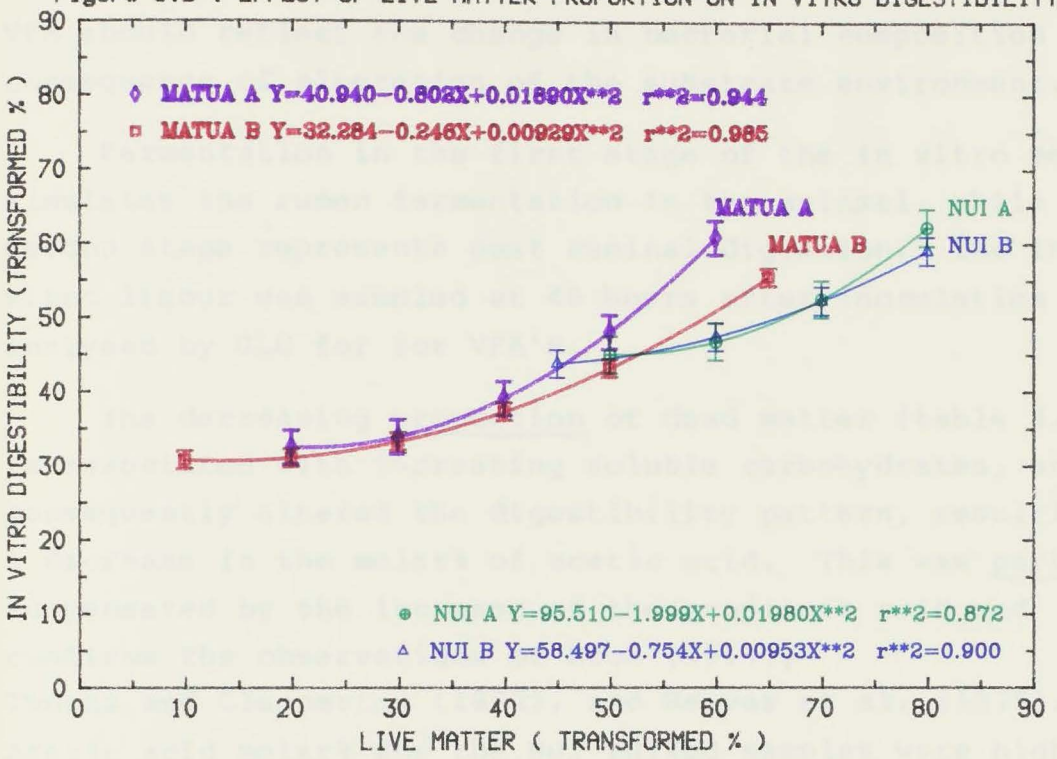
However as the amount of live material available increased towards the end of the collection period, the coefficient of variation improved considerably. This variation also followed through to the VFA determinations. Although the Matua IVD and the yield rose to similar values as Nui at the end of the collection period, there was a considerable lag, and the rise did not occur until spring.

Despite a cultivar having a high yield, it can only be utilized if it is digestible, and therefore a a nutritive value index was obtained by multiplying the IVD and the available dry matter. The lag in both the IVD and the growth of Matua, considerably reduced its nutritive value in comparison to the Nui swards. (see table 4.3.3B)

5.3 VOLATILE FATTY ACIDS

The fermentation of plant material is a progressive and sequential process. Initially the bacteria penetrates the intercellular space, then some species rupture the plant cell walls by cellulose digestion. Subsequently other species colonize the plant cell compartment to digest the

Figure 5.2 : EFFECT OF LIVE MATTER PROPORTION ON IN VITRO DIGESTIBILITY



cell contents. The bacterial population develops a stable composition of species which adhere to, and digest a particular polymer. This bacterial composition may change when that polymer is reduced or the pH is no longer favourable. Therefore the production and composition of the VFA should reflect the change in bacterial composition as a consequence of alteration of the substrate environment.

Fermentation in the first stage of the in vitro method simulates the rumen fermentation in the aninmal, while the second stage represents post ruminal digestion. The in vitro liqour was sampled at 48 hours after inoculation and analysed by GLC for for VFA'S.

The decreasing proportion of dead matter (table 4.2D) is associated with increasing soluble carbohydrates, and consequently altered the digestibility pattern, resulting in a decrease in the molar% of acetic acid. This was partially compensated by the increase of the propionic acid and confirms the observations of Rook (1964), Thomas and Clapperton (1972), and Beever et al. (1975). Acetic acid molar% for the Nui bulked samples were higher at the beginning of the sampling period when the sample was composed mainly of dead matter. The molar% of acetic and propionic acids are given in table 4.4C

The differences between the cultivars reflect on the difference in proportions of soluble carbohydrates during regrowth. The changes are more readily apparent for propionic acid, and this is consistent with the findings of Beever et al. (1975) that the digestible portion of cellulose and hemicellulose changed less than the soluble carbohydrates. In addition, starting dates showed a difference for Matua acetic acid. Loss of soluble carbohydrates in the dead material on the ground after topping may explain the peak for the acetic/propionic acid ratio at week 6. This corresponds to the lowest yield and IVD. Lowest total VFA also occurred at this time for both cultivars.

The following tables 5.3A and 5.3B show the results found by other authors.

Table 5.3A: Roughage diets
Thomas and Clapperton (1972)

acid	molar%
acetic	65-74
propionic	15-19
butyric	8-16

Table 5.3B:
Production of VFA from perennial ryegrass in the rumen
Beever,Cammell,Terry and Thompson (1975)

	spring	autumn
water sol. CHO	19.3	12.4%
nitrogen	1.6	2.2%
rumen VFA production-rate	5.1	3.9 moles/day
CHO conversion efficiency	1.7	1.6 moles VFA per mole CHO fermented
acetic acid	67	70 molar%
propionic acid	26	22 molar%
butyric acid	7	8 molar%

The blank samples, although without any substrate, contributed about 50-60 mg to the total VFA's determined. This arose from three sources:

1. VFA present in the inoculum of rumen liqour when mixed with the buffer
2. very small particles of digesta passing through the muslin cloth when the rumen fluid was strained.
3. fermentation of the glucose in the buffer

Most of the change of total VFA was contributed by acetic acid followed by propionic acid and to a lesser extent from butyric acid. The other acids: iso-butyric, iso-valeric, valeric and 2-methylbutyric acids were produced in only minor quantities, and contributed very little to the increase. 2-methylbutyric acid was detected in all the fermentations, and was about the same concentration of iso-valeric acid (3-methylbutyric acid).

Table 5.3C below provides a comparison between the composition of VFA's from in vitro grass hay, in vitro lucerne hay and the rumen fluid collected from sheep fed lucerne hay.

Table 5.3C : VFA molar% from 3 sources

	Ac	Pr	I-Bu	Bu	I-Val	Val
IVD grass hay	61.0	27.7	1.0	8.3	0.6	1.4
IVD lucerne	64.5	24.4	1.1	6.6	0.7	2.0
rumen fluid	62.5	16.0	4.5	12.7	2.3	2.1

acids
ac = acetic
pr = propionic
i-bu = iso-butyric
bu = butyric
i-val = iso-valeric
val = valeric

Lucerne hay being higher in protein than grass, may explain produced larger proportions of branched and higher chain VFA's.

The rumen fluid analysed was taken from sheep starved for 24 hours prior to rumen fluid collection. It was made up as artificial saliva inoculant before analysis by GLC. The starvation period may have substantially altered the VFA composition due to the different rates of VFA absorption from the rumen. The composition of the fluid was substantially lower in propionic acid and had higher concentrations of the branched chain and higher VFA's. This may be explained by the influence of several factors:

1. different rates of VFA absorption from the rumen
2. secondary fermentation of propionic acid into branched chain and higher acids.
3. soluble carbohydrates are rapidly and completely fermented to produce mainly propionic acid. However acetic acid is produced mainly from the slowly digested structural carbohydrates, which are still undergoing fermentation at the time of liqour collection. Because fermentation of soluble carbohydrates is complete, absorption from the rumen depleted the propionic acid in the rumen fluid.

5.4 EVALUATION OF GLC PACKING MATERIALS

An improvement in the peak shape after washing Porapak QS with acetone was reported by deSouta et al, (1975). Chromosorb 101 is also a porous divinylbenzene polymer and therefore washing with acetone was tried. This resulted in a shorter period required for conditioning the column and sharper peaks. This may be due to the acetone removing the residual volatiles remaining from manufacture.

The integration baseline level increased from including formic acid vapour (AR) into the carrier gas, and this has also been noted by Cochrane (1972). Inclusion of distilled water to the formic acid (AR) for a 95% solution almost completely eliminated the baseline dip. This supports the postulate of Schafer (1975), that water arriving in the carrier gas may lower the flame temperature and desensitize the flame ionisation detector. White and Leenheer (1975) reported that 3% orthophosphoric acid added to Porapak Q improved peak shape and separation. However, this could not be reproduced. This suggests that results for packing materials may not be reproducible between laboratories. This may be due to differences between batches of packing material and/or factors not mentioned in the reports.

Ottenstein and Bartley (1971) reported that teflon supports produced good peak symmetry and resolution, but had low efficiency requiring a longer column. The combination of a teflon support with a liquid phase to increase HETP may be worthy of investigation.

5.5 CONCLUSIONS

1. Nui sward samples had higher yield, higher grass% and clover%, and lower dead matter%. The Nui sward had more rapid and greater density of tillering, and higher clover plant density. The above occurred throughout the 20 week collection period. The start date did not affect the yield within a cultivar, but was significant for grass% and dead matter%. The increase of the live matter over the collection period was rapid for the Nui sward, but there was a 14 week lag before the Matua sward achieved the same rate of increase. The proportion of dead matter decreased rapidly for Nui but only slowly for the Matua samples. The defoliation to soil level was more suitable to the growth characteristics of the Nui plant.

2. IVD was higher for the Nui sward samples throughout the collection period. The Nui IVD rose rapidly after week 10 and reached a plateau at week 18. The Matua IVD also started rising at week 10, converged towards the Nui IVD values and had almost attained the same digestibility by week 20. The starting date for the trial was a significant factor. A start date one week later produced lower IVD for both Matua and Nui samples. The relationship between IVD and live matter% is not linear, IVD increased more rapidly than live matter%.

   The nutritve value index is a better indicator of pasture potential than IVD alone, since it also accounts for pasture yield. This showed that the Nui sward had about 2 times the amount of digestible material of the Matua sward through most of the collection period.

3. VFA analysis revealed that Nui samples produced greater amounts of total VFA and also that a later starting date resulted in a higher total VFA production. The total amount of VFA produced increased as the portion of substrate available for fermentation increased and was highly correlated to the IVD. The acetic/propionic acid ratios were significantly different between periods of regrowth, but were not significant between cultivars. The highest ratio corresponded to the lowest IVD and also to the lowest yield.

   The composition of VFA's changed between the periods of regrowth. Acetic acid (molar%) decreased while propionic and all the other minor VFA's increased. In contrast to the other VFA's, the start date had a greater effect on the branched chained iso-butyric and iso-valeric acids at each collection date.

4. A combination of formic acid vapour introduced into the carrier gas on Tenax GC/6% FAL-M allowed GLC separation of VFA's to achieve repetitve accuracy. This combination was able to reduce the adsorption of acetic acid and improve precision from 12% c.v. to less than 1% c.v. Separation of VFA's by GLC can determine differences in composition and in total VFA's from in vitro fermentations of different substrates.

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APPENDIX A

CHOICE OF GLC PARAMETERS

True separation of two consecutive peaks is measured by resolution R. This measures both column and solvent efficiency and accounts for narrowness of peaks and separation between maxima.

A.1 COLUMN EFFICIENCY

1. particle diameter: small uniform particle diameter will improve column efficiency, usually 100/120 mesh for diatomaceous earths.

2. flow rates: plot HETP vs flow rate. Minimun HETP is optimum carrier gas flow rate.

   HETP = L/N

   where L = column length
   N = 16(X/Y)**2

   substituting

   HETP = L/16(X/Y)**2

3. carrier gas: for highest efficiency, a high molecular weight gas should be used. Using a low molecular weight gas such as helium will reduce analysis time.

4. amount of liquid phase: low liquid loadings of 1-10% have faster analysis time and lower temperature operation, but lower sample capacity.

5. temperature: lowering temperature improves resolution but increases analysis time. Lower temperature should be accompanied by a lower liquid loading.

6. column diameter: increased efficiency obtained by decreasing internal diameter. 3 mm or 2 mm i.d. preferred.

A.2 DETERMINATION OF OPTIMUM OPERATING CONDITION

1. separate i-C4/C4 pair of peaks at 10,20,30,40,50,60 ml per minute carrier gas flow rate.

2. calculate HETP using HETP = L/16(X/Y)**2

3. graph HETP vs flow rate and find minimum for optimum flow rate

4. for shortest retention time, increase temperature until limits of resolution is reached. This must be within the operating range of the liquid phase.

APPENDIX B

GLC SEPARATION OF VFA's

B.1 EQUIPMENT AND MATERIALS

B.1.1 GLC

varian aerograph series 2800 fitted with a formic acid vapouriser and a bypass valve.
glass column 2400mm * 2mm i.d.
Tenax GC/6% FAL-M
flame ionisation detector 10^{-11} amps/mV

B.1.2 Data Processor

Shimadzu Chromatopac CR-1A
output in mg/100ml
optional: output from multiple calculation in mmoles/l

B.1.3 Acids (analar)

Crotonic (internal standard)
acetic, propionic, iso-butyric, butyric,
iso-valeric, 2-methylbutyric, valeric.
formic, orthophosphoric (H_3PO_4), Chromic.

B.1.4 Miscellaneous

1.0000 ± 0.0001 ml automatic dispenser
5.00 ± 0.01 ml automatic pipette
25 ul Hamilton syringe #81
30 ml screw cap polypropylene tubes
MSE benchtop centrifuge

B.2 DATA PROCESSOR PARAMETERS

see chapter 4, table: 4.5.3A to table: 4.5.3 F

B.3 GLC PARAMETERS

Table 1

gas flow (ml/min)		temp. (°C)		regulator press. (psi)
nitrogen	40	injector	130	65
hydrogen	40	column	140	50
air	400	detector	180	50

B.4 METHODS

B.4.1 Internal Standard Solution (200mg/100ml)

1. weigh 1000mg crotonic acid into a 500 ml volumetric flask.
2. add 20.0ml concentrated orthophosphoric acid (H_3PO_4) and make to volume.

B.4.2 Preparation Of Calibration Standard VFA

Standard VFA sample concentrations were made to reflect the free fatty acid from anaerobic fementations. Stock solutions of all acids were made to 1000mg/100ml.

Table 2: volumes to make calibration standard

acid	to make mg/100ml	stock solution required (ml)
acetic	250	25
propionic	125	12.5
i-butyric	20	2
butyric	50	5
i-valeric	20	2
2-methylbut.	20	2
valeric	20	2

1. from the stock solutions of 1000mg/100ml of the acids the required weight or volume equivalent were pipetted into the same 100ml volumetric fask. These volmes are shown in table 2.
2. make to 100ml with distilled water.

3. add 25.00ml of the internal standard solution made in the previous section.

B.5 SAMPLE PREPARATION

1. dispense 1.0000 ± 0.0001ml of internal standard solution into polypropylene tube.
2. pipette in 4.00 ± 0.01ml fermentation fluid
3. screw on cap and mix thoroughly
4. allow to stand for 30 minutes
5. centrifuge in an MSE benchtop centrifuge at 2000 rpm for 10 minutes.
6. (a) inject 0.2ul of supernatant onto GLC column.
   (b) if frozen, thaw to room temperature, mix well and centrifuge before analysis.

B.6 INJECTION TECHNIQUE

1. injection port, column and detector temperatures should have been stabilized.
2. before analysis inject on 25ul of 50% formic acid/distilled water to clear the column.
3. set GLC amplifier balance to zero
4. set level to <±1000 by amplifier bucking control
5. carry out S-TEST to automatically set the slope for the data processor
6. inject 0.2ul of standard sample to automatically calibrate the response factors for the data processor by the CALIB 1 command.
7. inject samples for analysis
8. periodicaly inject 0.2ul of standard sample to check the system for stability by the response factors.

B.6.1 Optional Multiple Calculation For Output In Mmoles/l

1. change to file 2
2. enter NAME, TIME, and coresponding F value from column 6 of table 4.5.3F chapter 4.
3. change back to file 1 and set to METHOD 4043 for multiple calculation.

Notes

1. The system cannot be calibrated when METHOD is set for multiple calculation
2. The file for multiple calculation must be adjacent to, and 1 higher than the main file.

APPENDIX C

IN VITRO DIGESTIBILITY METHOD

C.1 PREPARATION

1. Samples dried and ground through a 1mm sieve
2. Weigh 60 samples of 500mg to 525mg into 125 ml conical flasks. Include 4 each of, lucerne standard, hay standard as batch controls, and 4 blanks.
3. Weigh chemicals for 3 litres of artificial saliva into an air tight container.
4. Measure 3 litre of water into the 5 litre dispenser flask, replace the automatic dispenser and then place into the water bath. Set time switch to turn on the water bath at 6.30 am. Set thermostat at 39 °C
5. Check for sufficient CO_2 in the cylinder.
6. Arrange for rumen fistulated sheep to be starved for 24 hours prior to rumen fluid sampling.
7. stabilize the incubator at 39 °C

C.2 STAGE ONE: FERMENTATION

1. Dissolve buffer chemicals into prewarmed 3 litre of water and then gas with CO_2 until pH 6.9 while in water bath. Wet samples with 3 ml of water from dispenser.
2. Collect rumen fluid in a 4 litre liver pail by lifting the rumen. Strain through 4 layers of muslin cloth into a prewarmed thermos until full to maintain anaerobic conditions.
3. Transfer 750 ml of rumen fluid into the artificial saliva. Add 3 ml of 4% $CaCl_2$ and mix uniformly. Check pH and adjust if necessary.
4. Place samples into the water bath,displace air from the flasks with CO_2 and dispense 50.0 ml of innoculant into each flask. Stopper, wipe off water from outside of flasks, and place into a fan circulated incubator at 39 °C
5. Digest for 48 hours, swirling flasks morning, noon and late afternoon to ensure uniform digestion of the samples. Take care not to deposit sample above the liquid level.

C.3 STAGE TWO: ACID PEPSIN DIGESTION

1. ADD 150ml of 2% w/v pepsin solution to 450ml of 20% v/v HCl and mix thoroughly. Add to each sample in 1ml, 2ml, and 5ml aliqouts for a total of 8 ml to reduce foaming.

2. Restopper flasks and digest for a further 48 hours at 39 °C. Swirl flasks morning, noon and late afternoon.

C.4 FILTRATION

1. Filter residue into sintered glass crucibles, preweighed with celite 545 filter aid. Pour off supernatant first and rinse out residue with hot distilled water. Any residue remaining in the flask should be scraped loose by a glass rod with a rubber covered end, and rinsed into the crucible.

2. Place the crucible, with the residue into an oven at 105 °C. After 24 hours, allow the residue to cool in a dessicator, then weigh.

C.5 DIGESTIBILITY CALCULATION

$$IVD = [1-(res-bl)/diws]*100$$

where res = residue
bl = blank
diws = dry initial weight of sample

APPENDIX D

DATA

Matua A

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
2	*	10.7	2.1	80.4	31.2	133.8	1.33	0.45	2.90	*
2	*	0.8	1.5	97.6	30.3	133.3	1.31	0.44	2.95	*
2	*	2.3	4.8	92.2	25.4	131.1	1.31	0.44	2.95	*
2	*	0.8	4.2	83.0	30.8	133.3	2.34	0.89	2.63	*
2	*	2.5	3.8	91.8	34.0	133.3	1.96	0.79	2.46	*
2	*	0.0	0.0	100.0	*	*	*	*	*	*
4	833.3	30.5	1.3	68.2	35.6	122.4	2.06	0.86	2.39	296.6
4	1318.1	4.9	0.2	93.5	20.5	122.4	2.03	0.75	2.68	270.2
4	603.0	24.1	0.0	75.9	21.1	*	*	*	*	127.2
4	915.1	19.7	0.5	78.9	30.7	74.7	0.68	0.26	2.52	280.9
4	475.7	8.5	0.3	90.8	29.7	147.3	0.68	0.22	2.97	141.3
4	351.5	34.6	4.8	58.7	37.7	145.0	1.49	0.51	2.92	132.5
6	1021.2	*	*	*	14.5	94.6	0.98	0.29	3.30	148.0
6	1115.1	*	*	*	12.2	158.7	1.58	0.55	2.85	136.0
6	1412.1	*	*	*	10.4	*	*	*	*	146.8
6	724.2	*	*	*	14.9	108.8	1.06	0.41	2.54	107.9
6	1057.5	*	*	*	11.0	71.7	0.79	0.20	3.94	116.3
6	1318.1	*	*	*	16.3	141.0	1.34	0.52	2.56	214.8
8	603.0	34.7	0.7	54.3	19.0	144.3	0.36	0.13	2.71	114.5
8	1300.0	28.4	3.3	65.6	26.5	124.6	1.19	0.41	2.86	344.5
8	954.5	13.5	5.1	77.2	31.6	167.9	1.58	0.62	2.54	301.6
8	1457.5	36.8	7.9	51.2	24.6	140.3	2.58	0.90	2.85	358.5
8	890.9	29.0	8.4	47.3	25.0	144.3	0.34	0.14	2.35	222.7
8	842.4	*	*	*	26.5	144.3	1.36	0.49	2.73	223.2
10	1051.5	36.8	0.0	63.2	46.6	196.0	2.98	1.22	2.42	490.0
10	406.0	*	*	*	42.4	223.0	2.09	0.87	2.39	172.1
10	954.5	29.3	0.6	68.8	36.2	228.3	2.08	0.90	2.30	345.5
10	818.1	23.9	4.3	56.3	38.5	136.7	1.21	0.64	1.87	315.0
10	545.4	27.6	8.8	54.5	31.8	*	*	*	*	173.4
10	587.8	20.6	2.6	66.4	27.9	196.0	2.46	0.87	2.80	164.0

Matua A

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
12	1612.1	23.3	0.0	66.8	32.0	252.6	1.78	0.93	1.91	515.8
12	1148.4	39.7	0.0	60.3	33.7	236.8	2.11	0.76	2.74	387.0
12	709.0	32.5	0.0	65.2	39.4	250.5	2.24	0.87	2.56	279.3
12	1415.1	43.3	3.5	47.4	39.7	226.9	1.99	0.94	2.11	561.8
12	1351.5	35.9	1.1	40.8	49.9	296.3	2.66	0.90	2.94	674.4
12	1187.8	43.8	0.0	50.5	51.4	252.6	2.64	1.21	2.17	610.5
14	1381.8	38.5	1.8	55.7	53.6	271.7	2.38	1.14	2.07	740.6
14	1321.2	*	*	*	35.1	256.7	1.24	1.06	1.17	463.7
14	1518.1	*	*	*	58.5	261.0	2.28	0.64	3.52	888.1
14	1809.0	35.5	1.4	58.6	57.4	237.3	2.01	1.03	1.93	1038.4
14	1748.4	40.0	2.4	403	46.7	256.7	2.63	1.01	2.59	816.5
14	1863.6	29.1	1.7	53.8	49.1	256.7	2.69	1.17	2.29	915.0
16	1166.6	53.3	0.0	38.0	*	*	*	*	*	*
16	1712.1	57.3	0.0	41.2	53.3	275.9	1.18	0.45	2.57	912.5
16	2272.7	50.9	0.3	44.4	58.9	253.2	2.51	1.20	2.09	1338.6
16	1557.5	39.3	2.0	48.7	51.3	293.6	2.44	1.18	2.06	799.0
16	2045.4	51.8	1.4	39.1	55.1	280.9	2.06	1.12	1.84	1127.0
16	1378.7	42.7	2.3	48.8	*	*	*	*	*	*
18	2530.3	*	*	*	63.7	370.6	3.18	0.99	3.18	1611.8
18	2221.2	57.2	0.0	34.4	77.6	248.6	2.08	1.60	1.29	1723.6
18	2160.6	65.7	2.0	26.3	*	*	*	*	*	*
18	2048.4	54.2	1.5	22.9	63.0	241.3	3.59	1.09	3.28	1290.5
18	2330.3	56.7	3.0	29.9	69.5	363.0	3.13	1.10	2.82	1619.5
18	1781.8	61.5	0.4	28.2	65.9	328.3	2.81	1.60	1.75	1174.2
20	*	66.0	0.0	31.0	62.4	396.0	2.31	1.45	1.58	*
20	*	62.0	0.1	35.1	70.8	285.4	2.79	1.36	2.05	*
20	*	58.3	0.8	36.7	75.6	336.8	3.06	1.56	1.95	*
20	*	75.5	0.5	19.2	77.1	359.4	3.51	1.61	2.16	*
20	*	72.8	0.0	24.5	75.1	414.8	3.33	1.90	1.74	*
20	*	68.4	1.4	25.4	62.4	*	*	*	*	*

Matua B

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
2	466.6	1.2	0.0	98.8	26.5	191.7	2.51	0.99	1.95	123.6
2	845.4	8.2	0.1	91.7	34.0	180.7	2.74	1.01	2.71	287.4
2	1166.6	3.1	1.6	95.3	28.7	162.9	1.58	1.07	1.46	334.8
2	1124.2	6.6	1.3	92.2	29.3	*	*	*	*	329.4
2	927.2	5.4	1.7	92.1	25.0	153.7	1.53	0.90	1.69	231.8
2	1227.2	3.9	0.0	95.7	*	*	*	*	*	*
4	445.4	15.3	6.4	78.2	24.9	180.4	3.19	1.47	2.17	110.9
4	1003.0	14.5	0.3	85.2	25.1	203.8	1.88	1.18	1.58	251.7
4	890.9	15.0	0.0	84.5	25.6	187.6	1.44	0.75	1.91	228.0
4	1472.7	12.9	0.7	86.4	25.0	204.6	1.88	0.52	3.57	368.1
4	848.4	5.9	1.6	90.5	24.8	159.8	2.10	1.10	2.31	210.4
4	1060.6	3.4	0.2	96.4	27.3	192.6	2.10	1.10	2.31	289.5
6	384.8	13.3	0.0	83.7	15.7	205.7	1.89	0.66	1.85	60.4
6	675.7	7.8	4.6	86.9	*	*	*	*	*	*
6	800.0	15.0	1.5	82.2	18.9	216.5	1.96	0.67	2.91	151.2
6	815.1	6.3	3.3	90.4	16.7	*	*	*	*	136.1
6	787.8	13.6	0.6	85.2	*	*	*	*	*	*
6	1157.5	19.5	2.8	77.7	14.3	*	*	*	*	165.5
8	1318.1	16.6	2.1	80.2	21.5	180.2	1.74	0.62	2.80	283.4
8	648.4	14.3	0.0	85.7	13.7	64.9	0.61	0.59	1.03	88.8
8	1103.0	27.6	2.6	68.9	14.9	83.6	0.86	0.18	4.58	164.3
8	851.5	33.4	8.1	58.5	21.1	*	*	*	*	179.6
8	1603.0	16.5	10.8	69.7	15.5	*	*	*	*	248.4
8	2009.0	*	*	*	25.9	282.8	1.91	0.40	2.80	520.3
10	703.0	12.6	0.0	86.9	16.3	199.7	1.84	0.70	2.63	114.5
10	1303.0	11.0	1.1	70.2	26.0	104.9	1.04	0.64	1.61	338.7
10	1048.4	7.6	2.0	90.4	13.2	52.9	0.54	0.29	1.85	138.4
10	1257.5	17.4	1.0	81.2	17.7	186.1	1.14	0.55	2.07	222.5
10	884.8	8.7	5.5	74.4	18.2	121.7	1.18	0.58	2.03	161.0
10	436.3	9.9	0.4	88.8	19.6	64.5	1.12	0.55	2.03	85.5

Matua B

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
12	1169.7	21.9	0.3	77.5	32.6	*	*	*	*	381.3
12	1263.6	10.3	2.6	66.2	35.1	84.7	0.68	0.44	1.53	443.5
12	800.0	34.7	0.5	54.6	38.0	*	*	*	*	304.0
12	1239.3	23.5	1.7	64.7	40.6	124.2	1.06	0.91	1.16	503.1
12	1090.9	12.0	3.3	52.9	41.4	224.9	1.89	0.59	3.19	451.6
12	1033.3	13.8	0.6	78.2	*	*	*	*	*	*
14	645.4	39.5	0.0	59.0	33.9	134.3	1.21	0.47	2.57	218.8
14	1393.9	38.7	2.9	53.6	40.2	150.5	1.31	0.49	2.63	560.3
14	972.7	33.7	1.3	41.2	*	*	*	*	*	*
14	1336.3	20.6	0.0	76.2	35.3	160.8	1.36	0.44	3.06	471.7
14	1396.9	41.1	3.5	46.6	43.6	216.5	1.84	0.64	2.85	609.0
14	1593.9	24.2	3.1	68.7	34.7	188.2	1.61	0.85	1.89	553.1
16	1209.0	48.5	0.0	34.1	41.8	233.3	1.88	0.70	2.68	505.4
16	1884.8	47.6	1.9	32.9	40.8	254.0	2.16	0.87	2.46	769.0
16	2324.2	36.1	3.7	43.2	*	*	*	*	*	*
16	1496.9	46.2	2.2	46.5	45.4	*	*	*	*	679.6
16	1636.3	40.7	4.9	23.1	59.9	295.9	2.49	0.86	2.89	980.1
16	1757.5	33.3	0.3	45.0	56.1	201.4	1.73	1.18	1.45	986.0
18	1793.9	79.3	1.1	17.8	73.9	287.9	1.74	0.79	2.20	1325.7
18	975.7	75.6	0.9	11.6	67.6	300.1	1.84	0.82	2.20	659.6
18	3606.0	57.2	0.3	16.7	70.1	353.9	2.70	0.82	3.29	2527.8
18	2466.6	65.5	0.7	23.6	71.1	*	*	*	*	1753.8
18	2790.9	58.0	1.6	15.9	66.7	*	*	*	*	1861.5
18	2296.9	69.5	7.5	18.6	68.1	*	*	*	*	1564.2

Nui A

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
2	*	30.5	24.7	42.1	48.0	*	*	*	*	*
2	*	*	*	*	56.4	221.6	2.91	0.86	3.37	*
2	*	30.0	15.4	49.8	47.2	298.8	2.86	1.21	2.35	*
2	*	41.9	39.0	19.1	55.0	261.6	3.13	1.10	2.82	*
2	*	29.3	18.3	5.3	48.6	264.2	2.49	1.25	1.98	*
2	*	35.6	55.9	23.3	63.7	261.6	3.56	1.05	3.38	*
4	751.5	57.3	23.1	19.6	52.9	257.8	1.46	0.59	2.46	397.5
4	512.1	43.8	16.2	28.1	51.9	257.8	3.24	1.49	2.16	265.7
4	936.3	35.9	30.0	32.9	58.3	211.4	2.11	1.21	1.74	545.9
4	678.7	33.8	28.6	35.1	53.6	*	*	*	*	363.8
4	730.3	31.8	31.0	34.6	46.8	257.8	3.34	1.39	2.40	341.7
4	645.4	*	*	*	53.9	304.1	2.91	1.37	2.11	347.9
6	957.5	*	*	*	24.6	126.6	1.04	0.51	2.04	235.5
6	984.8	*	*	*	30.1	147.6	1.48	0.49	2.96	296.4
6	948.4	*	*	*	51.6	329.9	3.11	1.13	2.74	489.4
6	1078.7	*	*	*	36.9	230.7	2.23	1.21	1.83	398.0
6	896.9	*	*	*	27.2	227.3	2.16	0.85	2.54	243.9
6	896.9	*	*	*	21.7	*	*	*	*	194.6
8	636.3	71.8	21.1	0.0	63.0	261.6	2.41	1.00	2.40	400.9
8	981.8	65.2	23.5	0.0	60.7	129.9	1.13	0.47	2.39	595.9
8	1060.6	45.5	20.3	33.8	51.4	249.9	2.28	1.01	2.25	545.1
8	842.4	37.8	61.3	0.0	57.8	331.0	3.13	0.99	3.13	486.9
8	878.7	62.7	36.3	0.0	51.7	177.4	1.54	1.20	1.28	454.3
8	1415.1	50.8	34.3	0.0	52.0	285.8	2.74	0.76	3.57	735.8
10	636.3	59.5	26.1	0.0	65.2	285.2	2.16	1.05	2.05	414.9
10	1251.5	74.3	21.6	0.0	64.9	201.8	2.14	1.02	2.09	812.2
10	1503.0	80.6	13.0	0.0	47.3	285.1	2.61	0.87	2.97	710.9
10	963.6	64.9	32.9	0.0	66.4	368.4	3.86	1.03	3.71	639.8
10	1045.4	78.6	31.4	0.0	71.6	285.2	3.58	1.56	2.28	748.5
10	966.6	55.1	34.9	0.6	61.9	285.2	3.69	1.45	2.53	598.3

Nui A

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
12	1651.5	74.2	14.6	4.6	59.2	251.3	2.14	1.45	1.47	977.7
12	1881.8	64.3	13.5	12.8	66.6	282.3	3.56	1.05	3.38	1253.2
12	1527.2	84.8	8.9	3.7	62.8	270.7	2.34	1.55	1.51	959.1
12	1500.0	53.6	30.8	13.5	66.3	316.7	2.83	1.10	2.55	994.5
12	1612.1	*	*	*	72.4	319.0	2.74	1.24	2.21	1167.1
12	1760.6	*	*	*	59.0	253.8	2.21	1.34	1.64	1038.7
14	1651.5	83.6	9.1	3.6	*	*	*	*	*	*
14	2003.0	71.3	22.2	3.1	68.8	290.8	2.53	1.03	2.43	1378.0
14	1663.6	74.8	13.9	5.8	67.8	364.6	3.24	1.28	2.53	1127.9
14	1330.3	68.5	23.9	3.0	61.6	286.1	2.56	1.47	1.74	819.4
14	1151.5	68.6	20.6	5.0	*	*	*	*	*	*
14	2039.3	67.7	23.8	2.9	70.6	357.0	3.04	0.72	4.18	1439.8
16	2381.8	78.1	11.8	4.9	74.8	376.3	0.99	0.56	1.76	1781.6
16	*	84.7	8.3	4.9	73.9	406.5	3.56	1.53	2.31	*
16	1972.7	59.5	23.8	6.4	73.6	298.7	2.51	1.67	1.50	1451.9
16	2587.8	71.5	24.1	3.3	82.6	423.5	3.64	1.30	2.78	2137.5
16	2618.1	72.4	21.5	2.7	75.3	376.3	1.61	1.80	0.89	1971.4
16	2303.0	71.8	19.0	4.0	68.8	376.3	2.26	0.91	2.46	1584.4
18	2996.9	74.6	19.4	4.2	79.7	387.1	1.46	1.29	1.13	2388.5
18	3090.9	77.6	12.6	3.5	77.3	315.1	2.94	0.79	3.70	2389.2
18	2818.1	73.8	14.5	2.8	85.7	387.0	3.41	1.55	2.19	2415.1
18	2796.9	69.0	26.2	3.0	59.1	459.0	2.84	1.68	1.68	1653.0
18	2681.8	74.1	17.8	2.0	81.1	387.1	3.19	1.29	2.46	2174.9
18	2196.9	74.5	17.0	3.0	75.8	387.1	2.89	1.63	1.77	1665.3
20	*	81.2	11.9	6.0	77.6	280.8	2.48	1.52	1.62	*
20	*	73.4	15.4	7.3	79.5	413.0	3.69	1.24	2.97	*
20	*	69.5	20.4	6.2	81.6	381.2	3.93	1.80	2.17	*
20	*	65.0	29.6	3.4	58.3	412.9	3.61	1.86	1.93	*
20	*	64.5	28.0	7.1	81.6	381.2	4.17	1.68	2.47	*
20	*	59.9	31.9	5.0	71.2	417.9	3.53	1.86	1.89	*

Nui B

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
2	860.6	44.0	8.5	47.1	*	*	*	*	*	*
2	*	31.2	1.6	67.2	46.7	366.9	3.44	1.28	2.68	*
2	1263.6	31.4	17.6	48.1	45.3	301.5	2.79	1.05	2.65	572.4
2	*	21.2	31.8	44.9	57.8	352.9	3.24	1.21	2.67	*
2	1730.3	14.0	46.5	39.5	*	*	*	*	*	*
2	1100.0	34.0	26.0	40.0	*	*	*	*	*	*
4	739.3	49.8	22.4	24.7	52.0	351.2	3.28	1.30	2.50	384.4
4	1218.1	51.6	21.5	22.4	53.6	232.2	2.29	0.85	2.70	652.9
4	1203.0	31.8	26.0	35.8	61.7	406.4	3.78	1.43	2.65	742.2
4	1293.9	39.7	29.6	27.4	47.5	276.7	2.71	0.95	2.83	614.6
4	966.6	35.5	30.3	32.1	52.3	346.2	3.24	1.21	2.67	505.5
4	1133.3	51.9	26.4	19.3	54.7	363.7	3.33	1.30	2.54	619.9
6	754.5	29.9	23.6	38.4	43.5	294.9	2.78	0.94	2.94	328.2
6	545.4	20.0	20.7	53.1	*	*	*	*	*	*
6	539.3	39.7	35.2	24.4	*	*	*	*	*	*
6	763.6	36.3	36.8	25.1	*	*	*	*	*	*
6	921.2	27.4	35.5	32.5	*	*	*	*	*	*
6	951.5	39.7	37.5	20.6	*	*	*	*	*	*
8	827.2	53.7	14.3	31.4	47.2	218.2	2.14	0.74	2.89	390.4
8	1048.4	34.9	31.3	30.9	*	*	*	*	*	*
8	1012.1	46.4	29.9	12.7	*	*	*	*	*	*
8	587.8	49.1	20.0	19.3	*	*	*	*	*	*
8	875.7	34.2	28.6	32.9	46.3	258.7	2.56	0.85	3.01	405.4
8	1342.4	33.0	37.3	28.6	44.3	345.9	3.26	1.14	2.84	594.6
10	1324.2	71.3	26.7	0.0	57.1	358.5	3.31	1.17	2.82	756.1
10	1060.6	45.1	19.0	27.4	64.5	421.9	3.86	1.40	2.75	684.0
10	803.0	51.8	24.9	20.4	61.3	360.0	3.08	1.09	2.81	492.2
10	1081.8	51.4	21.9	20.0	63.9	384.8	3.56	1.29	2.74	691.2
10	1215.1	45.9	30.4	20.0	62.7	348.3	3.23	1.24	2.60	761.9
10	1148.4	72.0	21.2	0.0	58.7	286.4	2.73	0.99	2.73	674.1

nui B

Wk	Yield	Gr%	Cl%	Dd%	IVD%	TVFA	Acet mmol	Prop mmol	Ac/Pr	NVI
12	1636.3	71.8	8.0	19.1	53.6	338.3	1.73	0.83	2.06	877.0
12	1339.3	56.8	21.5	12.0	58.7	*	*	*	*	786.2
12	1712.1	64.4	19.0	1.7	71.7	412.9	3.56	1.60	2.21	1227.5
12	1181.8	62.9	21.9	10.3	59.5	284.9	2.43	1.16	2.09	703.1
12	1348.4	54.3	35.7	5.5	*	*	*	*	*	*
12	1584.8	66.5	16.4	12.1	57.9	375.3	1.61	0.78	2.06	917.6
14	1809.0	73.0	19.6	5.3	69.6	382.7	3.26	1.49	2.17	1259.1
14	1818.1	59.1	22.7	13.8	67.1	*	*	*	*	1220.0
14	1545.4	59.5	34.5	2.8	68.9	321.1	2.83	1.24	2.27	1064.8
14	1563.6	71.6	21.0	2.7	69.1	311.8	2.73	1.22	2.22	1080.4
14	1396.9	56.5	17.4	10.2	*	*	*	*	*	*
14	1612.1	71.5	20.1	4.0	69.1	252.2	2.26	1.02	2.20	1113.9
16	2415.1	79.7	14.0	5.2	75.5	422.8	3.66	1.71	2.13	1823.4
16	2412.1	58.5	31.1	5.5	73.2	389.3	3.39	1.51	2.24	1765.6
16	2496.9	64.3	30.9	2.5	76.5	320.2	2.76	1.26	2.17	1910.1
16	1890.9	55.3	31.6	3.6	*	*	*	*	*	*
16	2545.4	68.8	23.9	5.5	73.9	*	*	*	*	1881.0
16	3272.7	66.0	25.6	5.1	70.4	*	*	*	*	2304.0
18	2412.1	58.6	24.7	3.8	63.5	330.9	2.84	1.29	2.19	1531.7
18	3072.7	72.0	18.1	3.9	73.6	358.9	3.08	1.43	2.15	2261.5
18	2678.7	48.0	41.7	5.7	63.1	353.1	3.13	1.30	2.39	1690.3
18	2681.8	69.0	20.6	2.1	70.3	*	*	*	*	1885.3
18	2942.4	55.0	3.0	0.9	79.0	*	*	*	*	2324.5
18	2645.4	68.7	18.3	2.8	79.6	*	*	*	*	2105.7

Wk = Week, Gr% = Grass%, Cl% = Clover%, Dd% = Dead Matter%,
IVD% = In Vitro Digestibility %, Acet = Acetic acid,
Prop = Propionic acid, Ac/Pr = Acetic/Propionic ratio,
NVI = Nutritive value index.

Comparison of Octanol treatment to Control

ANALYSIS OF VARIANCE ON IVD%

SOURCE	DF	MS	VR
Oct/Ctrl	1	14.3	0.71 ns
ERROR	6	20.2	

Sample	N	MEAN	STDEV
Control	4	58.17	5.64
Octanol	4	55.50	2.93

POOLED STDEV = 4.49

* significant P(<0.05)
\+ " P(<0.01)
ns = not significant

Differences between In Vitro Digestibility Batches

ROW	IVD%	Luc/Hay	Batch
1	68.3	1	1
2	66.8	1	1
3	64.9	1	1
4	64.8	1	1
5	59.8	2	1
6	57.6	2	1
7	60.2	2	1
8	53.5	2	1
9	69.5	1	2
10	67.9	1	2
11	64.4	1	2
12	69.9	1	2
13	65.1	2	2
14	58.1	2	2
15	59.8	2	2
16	47.7	2	2
17	66.4	1	3
18	67.3	1	3
19	67.0	1	3
20	68.1	1	3
21	62.2	2	3
22	64.5	2	3
23	61.0	2	3
24	57.8	2	3
25	69.9	1	4
26	68.1	1	4
27	67.3	1	4
28	67.9	1	4
29	55.2	2	4
30	59.5	2	4
31	54.8	2	4
32	53.5	2	4

1=lucerne
2=hay

ANALYSIS OF VARIANCE ON IVD%

SOURCE	DF	MS	VR
Luc/Hay	1	686.4	62.40 * +
Batch	3	9.3	0.85 ns
INTERACTION	3	16.2	1.47 ns
ERROR	24	11.0	
TOTAL	31		

* significant P(<0.05)
\+ " P(<0.01)
ns not significant